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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Our general objective is to use completely defined cell-free transcription systems both to identify novel AR-associated cofactors and to investigate the mechanism of action of AR and both novel and previously identified candidate coactivators. To this end, we have expressed and purified 5 AR-associated cofactors and established a chromatin assembly system to study AR function with chromatin template. We have shown an enhancement of AR function by the TRAP/Mediator coactivator complex that is generally utilized by a number of different nuclear receptors. Relevant to the mechanism involved, we have identified two TRAP/Mediator subunits as potential targets for the liganded AR receptor. The expression of AR and 10 cofactors by quantitative in situ RNA hybridization in 44 primary prostate cancers with different degree of differentiation was investigated. Our results revealed near constant expression of AR and heterogeneous expression of AR cofactors. Expression of PIAS1 and Ran/ARA24 was increased and expression of ELE1/ATRA70 was decreased. In addition, we demonstrated that the human prostate tumor cell proliferation and colony formation are markedly reduced by over-expression of ELE1/ARA70. Together, these findings indicate that the change of expression levels of AR cofactors may play important roles in prostate growth and tumorigenesis.

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INTRODUCTION

The androgen receptor mediates the biological functions of androgens in gene expression and is implicated in prostate cancer. Androgen ablation therapy, while effective in early androgen-dependent stages, nonetheless fails in the androgen-independent stages of advanced prostate cancer. Although the mechanism for the clinical response to androgen withdrawal therapy is not clear, androgen-independent progression has been associated with mutations or amplification of the androgen receptor gene and activation of intracellular signal transduction pathways that stimulate the androgen receptor function. Insights into this problem and a possible therapy for prostate cancer may be gained from a detailed understanding of the molecular mechanisms by which androgen receptor activates key target genes in malignant prostate cells. Our hypothesis is (i) that the transition from androgen-dependent to androgen-independent prostatic cancer involves alterations in the levels or activities of coactivators that interact physically and/or functionally with AR to activate genes whose expression contributes to malignancy and (ii) that an understanding of the nature, regulation and mechanism of action of these factors can facilitate the development of anti-prostatic cancer therapy. The general objective is to use completely defined cell-free transcription systems both to identify novel AR-associated cofactors, isolated by conventional fractionation and/or affinity methods, and to investigate the mechanism of action of these and previously identified candidate coactivators. Toward this objective we will (i) investigate purified androgen receptor and cognate cofactor functions in cell-free transcription systems reconstituted with general initiation factors and cofactors, wild type and mutant androgen receptors, and both DNA and chromatin templates, (ii) identify by complementation assays, purify by affinity methods, and characterize mechanistically additional (co)factors that act in association with androgen receptor on androgen receptor-activated genes and (iii) investigate possible changes either in the levels or in the activities (functional modifications) of these factors (e.g. in response to other signal transduction pathways) during prostate cancer development. This approach might provide new drug development insights into targeting the androgen receptor pathway downstream of the point of ling-receptor interaction.

BODY

Task 1 **To analyze the cofactor requirements and mechanisms of action of AR on purified DNA templates in a purified system (months 1-12):**

a. express and purify various cofactors (months 1-6).

b. immunopurify cofactor complexes from cell lines (months 1-6).

c. perform transcription assays (months 1-6).

Related to Task 1, we have established a well-defined cell-free transcription system comprised of the recombinant TFIIA, TFIIB, TFIIE, TFIIF, PC4 and the natural immuno-purified TFIID, TFIIH and RNA Pol II complexes (Appendix I). Using a synthetic ARE-containing DNA template, we have systematically analyzed requirements for the TRAP/Mediator components, SRC1, p300 and miscellaneous coactivators (ARA70, PGC-1 and AES). ARA70 (Figure 1A, lane 2), PGC-1 (lane 4), AES (lanes 6 and 7), SRC1 (lanes 9-11) and p300 (lanes 12-14) were expressed in and were purified from Sf9 or bacterial cells. AES strongly inhibited AR-dependent transcription both in vitro and in vivo (Appendix I). Thus we have identified a new negative regulatory pathway for AR through an associated co-repressor. SRC1, p300, ARA70 and PGC-1 failed to activate AR-dependent transcription in this reconstituted system, perhaps not surprisingly in view of demonstrated or presumed functions of many of these components at the level of chromatin. In contrast, the immuno-purified TRAP/Mediator complex (Figure 1A, lane 16) enhanced AR function on naked DNA templates (Figure 1B, lanes 3 and 4 versus lane 2) and this function was dependent on AR (data not shown). Protein-protein pull-down assays revealed that AR interacts with TRAP/Mediator complex in HeLa cell nuclear extract (Figure 2C, lanes 4 and 6). Subsequent assays with individually expressed TRAPs indicated that AR directly interacts with TRAP220 (Figure 2A, lanes 3 and 5) and with TRAP80 (lanes 8 and 10). These interactions were found to be ligand independent (Figure 2C, lanes 4 versus 6; 2A, lanes 3 versus 5; 8 versus 9), a somewhat surprising observation in view of the fact that several other nuclear receptors (TR, VDR, ER,.....) show ligand dependent interactions with TRAP220 through the AF2 domain (1). The interaction is independent both of the TRAP220 NR boxes previously implicated in ligand-dependent interactions with other receptors and of the ligand binding domain (LBD) of AR (Figure 2B, lanes 4 and 5 versus lane 3). These results indicate that AR may interact with one or more novel TRAPs through the AF1 domain, consistent with the observation that transactivation by AR is mediated mainly through its AF1 region and with the finding of ligand-independent interactions of TRAP170 with the AF1 domain of GR (2). Transfection assays with TRAP220$^{-/-}$ mouse embryo fibroblasts (derived from embryonic knockout mice, 3) also showed that TRAP220 is essential for optimal activation by AR lacking the LDB but (under these suboptimal assay conditions in non-prostate cells) not for activation by intact AR (Figure 3). These results thus implicate TRAP220 in a ligand-independent activation function of AR and are of potential significance with regard to androgen-independent growth of prostate cancer cells.

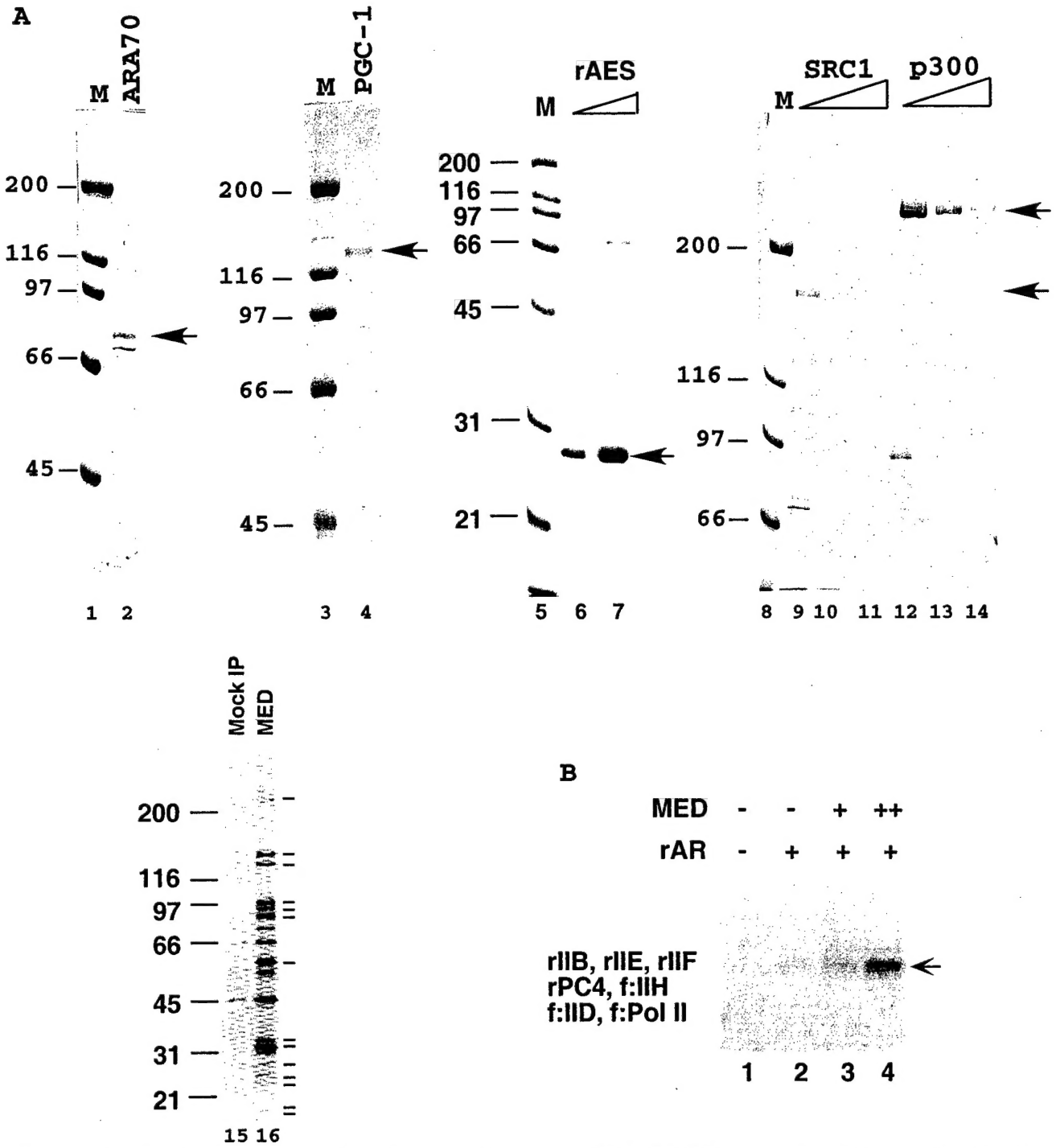

Figure 1. A. Purification of various cofactors. SDS-PAGE analysis of the purifed factors. The purified recombinant ARA70 (lane 2), PGC-1 (lane 4), AES (lanes 6 and 7), SRC1 (lanes 9-11), p300 (lanes 12-14) and the immunopurified TRAP/Mediator complex (lane 16) were loaded on SDS-gels and stained with Coomassie blue R250 (lanes 1-14) or with silver (lanes 15 to 16). The TRAP/Mediator complex was isolated from a cell line expressing FLAG-tagged Nut 2 subunit of the TRAP/Mediator complex (4) and the subunits ascribed to the TRAP/Mediator complex are indicated by short lines at the right. Lane 15 is control immunoprecipitation from HeLa cells. Lanes 1, 3, 5 and 8 show molecular weight markers (Bio-Rad). **B. The TRAP/Mediator complex enhances AR function.** A synthetic template containing 4 ARE elements is transcribed in the system reconstituted with purified factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, Pol II, and PC4), with additions of rAR and TRAP/Mediator complex . The specifically-initiated transcript is indicated by arrows and was monitored by primer extension.

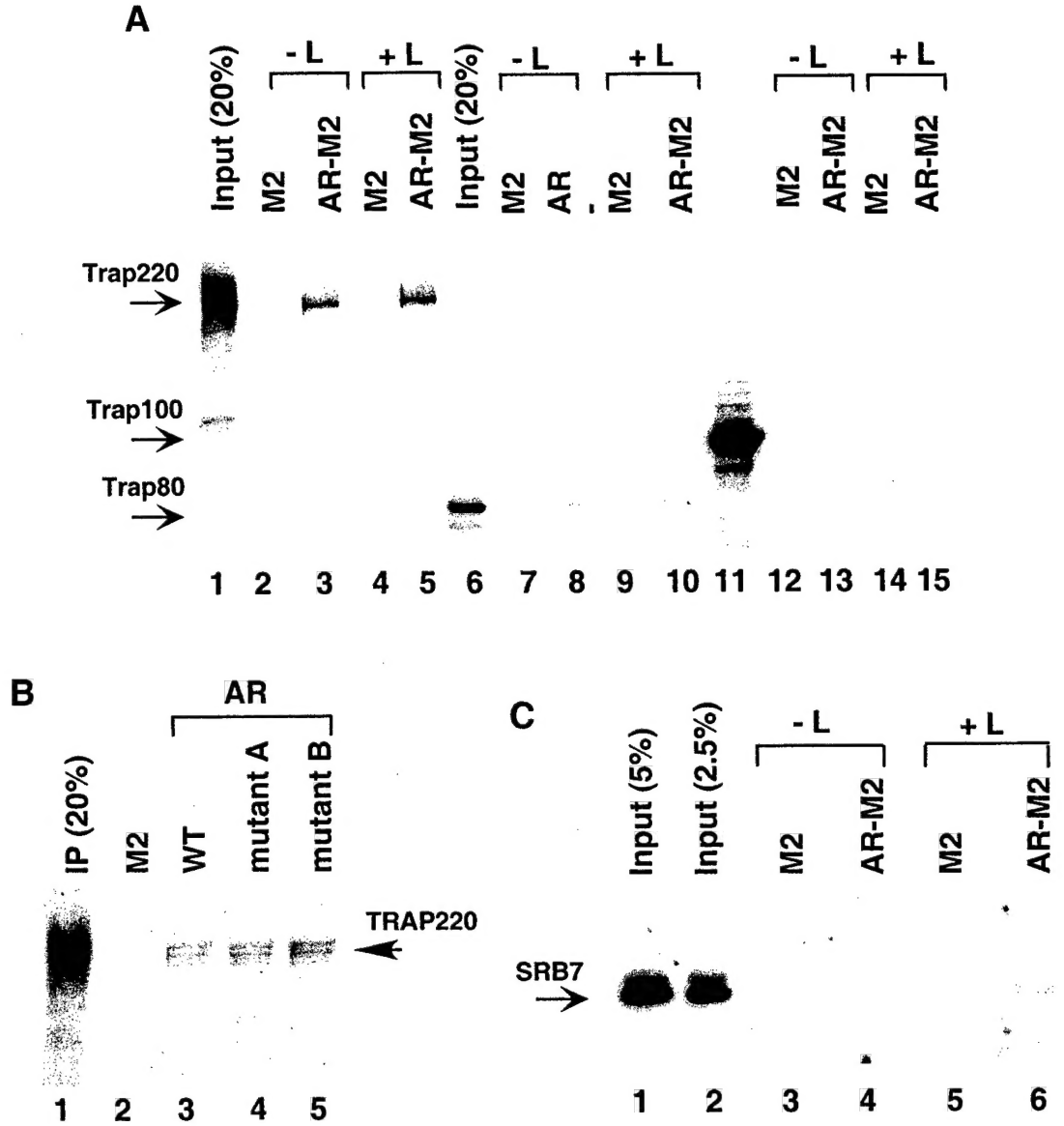

Figure 2. AR directly interacts with the TRAP220 and TRAP80. 100 ng of f:AR were immobilized on M2 agarose beads and incubated with 5µl of TNT rabbit reticulocyte lysates containing S^{35}-labeled TRAP80 (panel A, lanes 6-10), TRAP100 (panel A, lanes 11-15) or wild type TRAP220 (panel A, lanes 1-5; panel B, lane 3), nuclear receptor-interacting domain A mutant TRAP220 (panel B, lane 4) and nuclear receptor-interacting domain B mutant TRAP220 (panel B, lane 5), respectively, for 2 hrs at 4°C in BC150-0.1%NP40 in the presence of 100µM of R1881 (panel A, lanes 4, 5, 9 10, 14 and 15) or in the absence of R1881 (panel A, lanes 2, 3, 7, 8, 12 and 13). The beads were washed with the incubation buffer and analyzed by SDS-PAGE. Lanes 1 is 20% of input. **C. AR interacts with Mediator complex in nuclear extract.** 100 ng of f:AR were immobilized on M2 agarose beads and incubated with 50µl of nuclear extract from HeLa cells for 2 hrs at 4°C in BC150-0.1%NP40 in the presence of 100µM of R1881 (lanes 5 and 6) or in the absence of R1881 (lanes 3 and 4). The beads were washed with the incubation buffer and analyzed by Western blot with anti-SRB7 antibody. Lanes 1 and 2 are 5% and 2.5% of input, respectively.

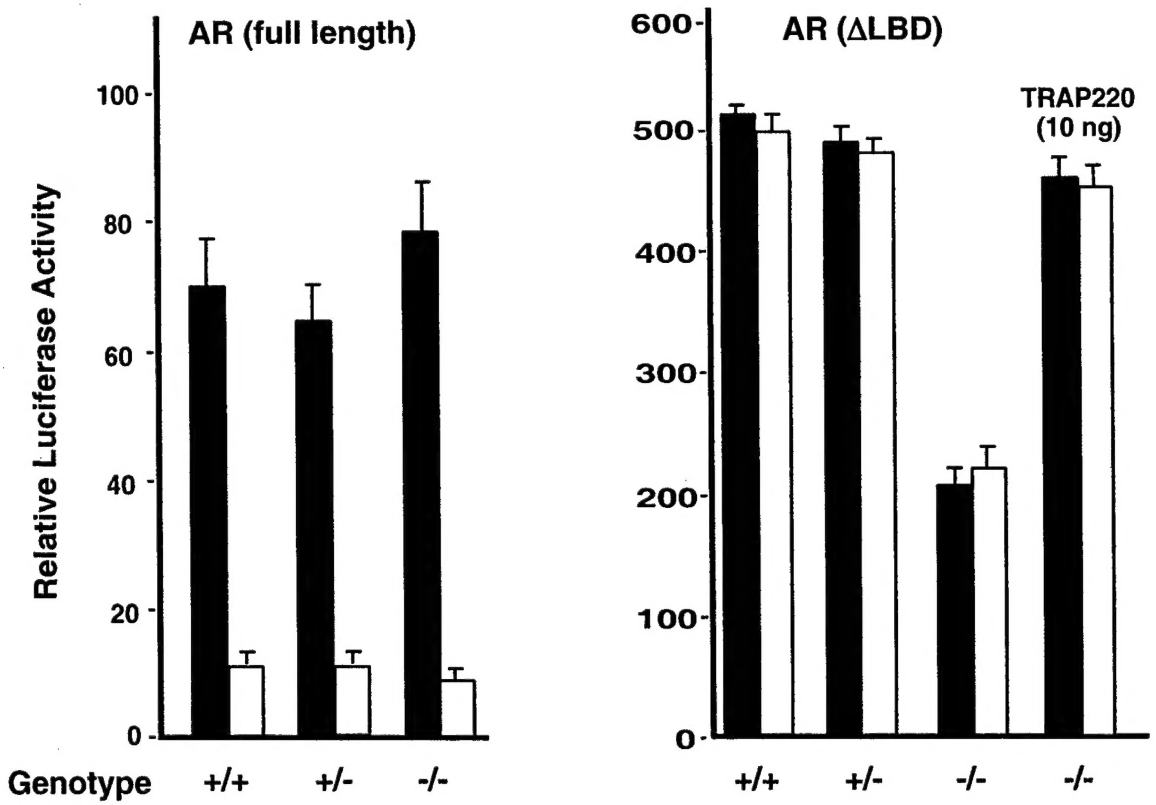

Figure 3. AR function in TRAP220 knockout MEFs (p53-/-).
AR(ΔLBD)-, not the full length AR-, driven transcription is affected in Trap220-/- MEFs (3). The MEFs of each genotype were transfected with 100 ng ARE-luciferase reporter, 10 ng control luciferase pRL-SV40, 30 ng human full length AR or AR(ΔLBD) and 10 ng TRAP220 construct as indicated. Cells were cultured in the absence or presence of ligand, R1881 (100 nM), and dual luciferase activities were measured 48 hr after transfection.

Task 2 **To analyze the cofactor requirements and mechanisms of action of AR on chromatin templates (months 1-12);**

a. purify human core histones from HeLa cells (months 1-6)
b. express and purify NAP1, ACF1 and ISWI (months 1-6)
c. assemble chromatin in vitro (months 6-12)
d. perform transcription assays on the chromatin template (months 6-12)

An analysis of transcription in the context of a more physiological chromatin template is especially relevant in light of increasing evidence for regulation at the level of chromatin remodeling, both by ATP-dependent remodeling factors (SWI/SNF, NURF etc.) and by HAT-containing coactivators (GCN5/PCAF, p300/CBP, p160 factors, etc.). Toward this goal for AR, we have established a chromatin assembly system based on purified components (Figure 4, lanes 1-7). In the initial analysis we have used a template in which flanking 5S DNA sequences position nucleosomes over the E4 core promoter and upstream 5 GAL4 binding sites (5). A chromatin template was assembled as described by Ito et al. (6). Transcription assays were conducted initially with the activator GAL4-VP16, recombinant p300, and HeLa nuclear extract as a source of general transcription factors. In this system transcription from the DNA template yields a high level (15-20 fold above basal) of activator-dependent transcription that is independent of added p300 and acetyl-CoA (Figure 4C, lanes 1-4). As expected, prior assembly of the DNA in chromatin nearly completely represses both basal activator-dependent transcription (lane 5). However, activator-dependent transcription from the chromatin template is dramatically enhanced by the simultaneous addition of p300 and acetyl-CoA (lane 8) and this activity was dependent on acetyl-CoA as well as p300 (lanes 7 and 9 versus lane 8). Hence, and especially in light of documented networks of nuclear receptor-p300-p160 interactions, this system is suitable for an analysis of the role of these and other cofactors in AR function.

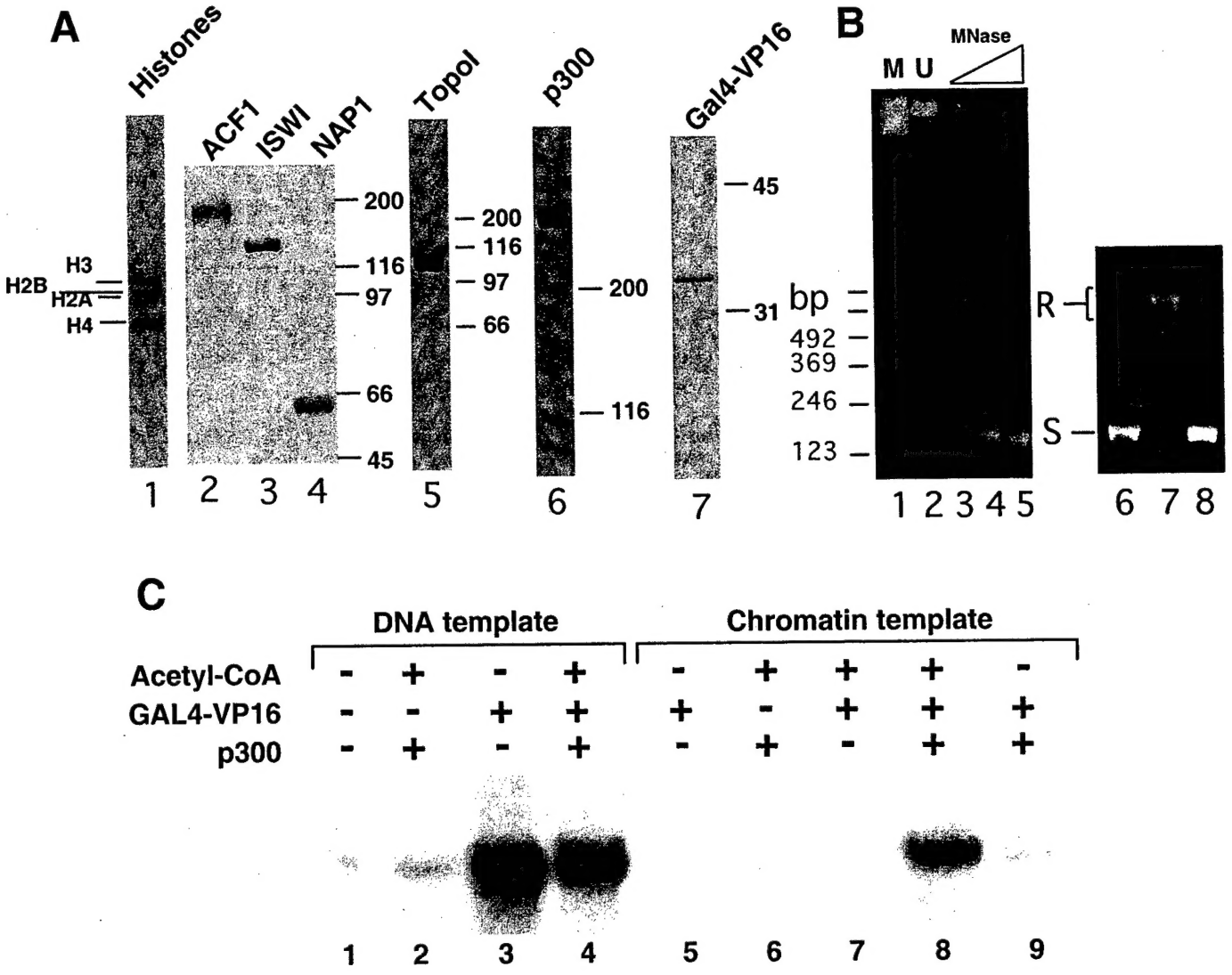

Figure 4. Activator-dependent transcription from a chromatin template mediated by coactivator p300.
A. SDS-PAGE analysis of purified factors. Human core histones were purified from HeLa nuclear pellet as described (5). Recombinant His-tagged nucleosome assembly protein 1 (NAP1) and transcription activator GAL4-VP16 were expressed in E. coli and purified on Ni-NTA agarose (Qiagen). FLAG-tagged ACF1, ISWI and Topo I and His-tagged p300 were expressed in Sf9 cells via baculavirus and purified on M2 agarose and Ni-NTA agarose, respectively.
B. Chromatin assembly and characterization. The plasmid pG5ML was assembled into a chromatin template with purified human core histones, Topo I, NAP1, ACF1 and ISWI as described (6). The in vitro assembled chromatin was characterized by MNase digestion analysis (lanes 1-5) and by DNA supercoiling assay (lanes 6-8). After treated with increaseing concentrations of MNase, the sample was deproteinization with proteinase K digestion and the resulting DNA was resolved on a 1.5% agarose gel and staining with ethidium bromide. Lane 1 is 123 bp DNA ladder. lane 6, supercoiled DNA (S) used for assembly; lane 7, relaxed DNA (R) after Topo I treatment of the DNA of lane 6; lane 8, supercoiled DNA isolated by deproteinization of assembled chromatin.
C. Transcription from DNA versus chromatin templates. GAL4-VP16 markedly activated transcription from the DNA template independently of p300 or acetyl CoA (lanes 1-4). By contrast, assembly of the DNA within chromatin drastically inhibited transcription in the presence of GAL4-VP16 (lane 5). However the addition of p300 and acetyl-CoA restored most of the activity with GAL4-VP16 (lane 8) and this activity was largely dependent on acetyl-CoA as well as p300 (lanes 7 and 9 versus lane 8).

Task 3 **To determine activators and cognate cofactors acting in conjunction with AR on natural promoters (months 1-12):**

a. **analyze the Probasin promoter elements (months 1-6)**
b. **express and purify the involved activators (months 6-12)**
c. **perform transcription assays using the recombinant activators (months 6-12)**

In order to study AR function in the context of natural promoters and synergistic interactions with other activators. We have determined the promoter elements on the probasin promoter and found that the Myb-binding site is a critical element. The recombinant Myb was expressed in bacteria and purified through Ni-NTA agarose column. In the purified reconstituted transcription system containing the purified general factors, cognate coactivator (PC4), and purified AR, we did not observed the synergism between AR and Myb. Chromatin templates may be necessary to elicit synergism.

Task 8 **Cell specificity and function of fAR-associated cofactors (months 30-36):**

a. **perform Western blotting analysis (months 30-36)**
b. **perform in situ hybridization with prostate cancer samples (months 30-36)**

Related to Task 8, we studied the expression of AR and 10 cofactors by quantitative in situ RNA hybridization in 44 primary prostate cancers with different degree of differentiation. Our results revealed near constant expression of AR and heterogeneous expression of AR cofactors. Expression of PIAS1 and Ran/ARA24 was increased and expression of ELE1/ATRA70 was decreased. Interestingly, ARA55 is only expressed in prostate stroma cells compared with the preferential epithelial expression of ARA160, ELE1/ARA70, ARA55, Ran/ARA24, and PIAS1. In addition, we demonstrated that the human prostate tumor cell proliferation and colony formation are markedly reduced by over-expression of ELE1/ARA70. Together, these findings indicate that the change of expression levels of AR cofactors may play important, yet distinct, roles in prostate growth and tumorigenesis. These results are included in a manuscript accepted by the American Journal of Pathology for publication (Appendix II).

KER RESEARCH ACCOMPLISHMENTS

1. TRAP/Mediator complex enhances the AR-dependent transcription both in vitro and in vivo.
1. Established a chromatin assemble system.
1. Found that change of expression levels of AR cofactors may play important roles in prostate growth and tumorigenesis.

REPORTABLE OUTCOMES

A manuscript (Appendix II) has been accepted for publication in the American Journal of Pathology

CONCLUSIONS

We have fulfilled the tasks proposed for period from July 1, 2001 to June 30, 2002. In addition, have done some work related to the task 8. The in vitro reconstituted transcription system and the chromatin assembly system provide an excellent base for future study of the AR and its cofactors in vitro. Our research indicated that the change of expression levels of AR cofactors might play important roles in prostate growth and tumorigenesis. Further study related the mechanism of their action would help to understand their function in prostate tumorigenesis and prostate cancer growth.

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Inhibition of Androgen Receptor-Mediated Transcription by Amino-Terminal Enhancer of split

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A yeast two-hybrid assay has identified an androgen-dependent interaction of androgen receptor (AR) with amino-terminal enhancer of split (AES), a member of the highly conserved Groucho/TLE family of corepressors. Full-length AR, as well as the N-terminal fragment of AR, showed direct interactions with AES in in vitro protein-protein interaction assays. AES specifically inhibited AR-mediated transcription in a well-defined cell-free transcription system and interacted specifically with the basal transcription factor (TFIIE) in HeLa nuclear extract. These observations implicate AES as a selective repressor of ligand-dependent AR-mediated transcription that acts by directly interacting with AR and by targeting the basal transcription machinery.

Androgen receptor (AR) is a member of the superfamily of ligand-inducible transcription factors and mediates the biological actions of androgens (19). Like other superfamily members, AR contains a central DNA-binding domain, a C-terminal ligand-binding domain with an associated AF-2 activation domain, and a large N-terminal region containing the AF-1 activation domain (4, 26). Nuclear receptors regulate the transcription of their target genes through the agency of various coactivators and corepressors that are recruited to target genes through interactions with promoter-bound receptors (56). Many of the known coactivators for nuclear receptors contain histone acetyltransferase activities and are thought to act mainly through targeted chromatin structural perturbations that facilitate the subsequent recruitment (to the promoter) and function of other transcriptional coactivators and basal transcriptional factors (3). Transcriptional corepressors, by contrast, mediate repression by various nuclear receptors. Some nuclear receptors (including retinoid receptor, thyroid hormone receptor, vitamin D receptor, and certain orphan receptors) that are not associated with heat shock proteins in their unliganded state repress transcription by recruitment of corepressor complexes (15, 35). Corepressor complexes contain histone deacetylase (HDAC) activities that maintain chromatin in a configuration that excludes functional interactions of the general transcriptional machinery with the promoter. In contrast, unliganded steroid receptors (including AR) generally associate with heat shock proteins and, upon ligand binding, dissociate from the heat shock proteins, translocate to the nucleus, and associate with coactivators to activate or repress target genes (30).

Another type of corepressor, implicated in the function of other types of repressors, is the Groucho/TLE family (see Fig. 1D) (5, 11). The larger family members such as *Drosophila* Groucho and its mammalian homologues, the TLE proteins (transducin-like enhancer of split [TLE1-3]), share five domains. A carboxyl-terminal WD-40 repeat domain (WD-40) and an amino-terminal glutamine-rich domain (Q) are highly conserved. In the much less well-conserved central region, there is a loosely conserved CcN motif (CcN), consisting of putative cdc2 kinase and casein kinase II phosphorylation sites, and two poorly conserved regions (GP and SP) that are characteristically rich in either glycine and proline (GP) or serine and proline (SP) residues. A shorter family member, human TLE4, is similar except for the absence of the amino-terminal Q and GP domains. The shortest family member, amino-terminal enhancer of split (AES), shares only the first two regions of the amino terminus.

The Q domain mediates both homo- and hetero-oligomerization between Groucho/TLE family proteins, whereas the WD-40 repeats appear to mediate protein-protein interactions with relevant DNA-binding activators and repressors. Groucho/TLE proteins do not have recognizable DNA-binding domains but can repress transcription directly if tethered to DNA through a Gal4 DNA-binding domain or if recruited to DNA through interactions with other DNA-binding activators and repressors. The function of AES remains controversial. It was suggested that AES might act as an inhibitor of Groucho/TLE corepressors by dominant negative mechanisms (28, 45). On the other hand, AES has been shown to mediate Blimp-1-dependent repression of the beta interferon gene (41) and to repress NF-κB-driven gene expression (51) in vivo.

Here we demonstrate that AES physically interacts with human AR both in vivo and in vitro and that it represses AR-dependent transcription both in transient-transfection assays and in a purified cell-free transcription system. In addition, we find that AES interacts selectively with the basal transcription factor TFIIE. These observations indicate that AES represses AR-driven transcription by directly targeting the basal transcription machinery.

MATERIALS AND METHODS

Yeast screening. The yeast two-hybrid screening was performed as previously described with minor modifications (57). Briefly, an expression plasmid encoding the Cyto-trap bait was generated by inserting the cDNA sequences of human AR into pSos, a yeast shuttle vector. *Saccharomyces cerevisiae* strain cdc25H was

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transformed sequentially with pSos-AR and human prostate cDNA library expression plasmids (Stratagene). The positive clones were those that grow on the plates with galactose and 100 nM R1881 at 37°C but not on galactose plates in the absence of R1881. Plasmids were rescued from each of these positive colonies and identified by nucleotide sequencing.

Mammalian two-hybrid analysis. Expression vectors that encode hybrid polypeptides were produced by inserting AES cDNA sequences into the pCMV-GAL4 vector or by inserting AR cDNA sequences into the pVP-FLAG7 vector (57). A mammalian two-hybrid assay was conducted in 293T cells as described previously (52), except that when indicated, transfected cells were incubated for 40 h with medium containing 100 nM R1881. The pRL-LUC plasmid was included in each culture of transfected cells as an internal control. The luciferase activity was determined using the Dual-luciferase assay system (Promega).

Transient transfection. The AR and AES expression vectors for transfection assays were constructed by inserting their corresponding cDNA sequences into pcDNA3.1. The AR-responsive reporter gene ARE4-LUC contains four AR-responsive elements ahead of the E4 basal promoter and the luciferase gene. HeLa Z cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Transfections were performed using SuperFect reagent (Qiagen). Briefly, 10^5 cells were plated onto 24-well plates approximately 24 h before transfection. After the plates were washed with phosphate-buffered saline, cells in each well were transfected with 50 ng of an expression vector (AR, estrogen receptor [ER], or thyroid hormone receptor [TR]), 100 ng of the reporter plasmid, 5 ng of the pRL-LUC internal control plasmid, and the indicated amount of the AES expression vector. The total amount of DNA was adjusted to 1 μg with pcDNA3.1. Transfections were conducted in phenol red-free RPMI 1640 medium, and 2 h later the medium was changed either to phenol red-free RPMI 1640 medium plus 10% charcoal dextran-stripped fetal bovine serum or to regular medium containing 100 nM R1881, 1 μM β-estradiol, or 10 nM T3. The cells were cultured for another 48 h and harvested for luciferase assays (Promega). For trichostatin A (TSA) treatment, 10 ng of TSA per ml was added to transfected cells 24 h before harvest. Three independent experiments were carried out in each case for statistical analysis.

Purification of transcription factors. Histidine-tagged TFIIAα and TFIIAγ were expressed in bacteria via the pRSET vector and purified on Ni-nitritotriacetic acid (NTA)-agarose in the presence of 6 M urea (8). TFIIAαγ was reconstituted with a combination of equimolar amounts of purified TFIIAα and TFIIAγ and dialyzed against BC300–0.1% NP-40. The FLAG-tagged TFIIAβ was expressed via vector pET15d and purified on M2 agarose. TFIIA was reconstituted with a combination of equal amounts of TFIIAαγ and TFIIAβ. Bacterially expressed histidine-tagged TFIIB was purified on Ni-NTA-agarose and phosphocellulose. Histidine-tagged TFIIEα and FLAG-tagged TFIIEβ were expressed in bacteria and purified on Ni-NTA-agarose and M2 agarose, respectively, and TFIIE was reconstituted with a combination of two subunits and further purified through M2 agarose. TFIIF was expressed and reconstituted as reported previously (53). Bacterially expressed untagged PC4 was purified through heparin-Sepharose and phosphocellulose (13). Histidine-tagged GAL4-VP16 was expressed in bacteria and purified through Ni-NTA-agarose and S-Sepharose.

Nuclear extract was made from the FLAG-tagged TAF135 cell line (31) and further fractionated by phosphocellulose and DEAE cellulose (DE52) chromatography. FLAG-tagged TFIID (f:TFIID) was isolated from the 0.3 M KCl fraction of a DE52 column by M2 agarose affinity purification. HeLa cell lines stably expressing FLAG-tagged RPB9 and FLAG-tagged XRB1 were established, and FLAG-tagged RNA polymerase II (f:PolII) and TFIIH (f:TFIIH) were purified from these cell lines by described procedures (54). Recombinant human androgen receptor was expressed in Sf9 cells via a baculovirus vector as a FLAG-tagged fusion protein and purified on M2 agarose.

In vitro transcription and primer extension. To create the template pARE-E4, a DNA fragment containing four copies of the androgen-responsive element (AGAACAGCAAGTGCT) from the PSA promoter was inserted into *Sph*I and *Xba*I sites of the vector pG5E4. Transcription reactions were carried out in a final volume of 25 μl, and the reaction mixtures contained 90 fmol of supercoiled plasmid DNA template, 20 mM HEPES (pH 7.9), 12% glycerol, 6 mM $MgCl_2$, 70 mM KCl, 5 mM dithiothreitol (DTT), 600 μM each ATP, UTP, CTP, and GTP, 40 U of recombinant RNasin, 0.5 mg of bovine serum albumin per ml, 12 ng of TFIIA, 30 ng of TFIIB, 2 μl of f:TFIID, 0.5 μl of f:TFIIH, 12 ng of TFIIF, 6 ng of TFIIE, 150 ng of PC4, 1 μl of f:PolII, 30 ng of human AR, and various amounts of different cofactors. After a 60-min incubation at 30°C, the transcription reactions were stopped by adding 175 μl of stop solution (1% sodium dodecyl sulfate, 5 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 20 μg of glycogen, 40 μg of proteinase K) and incubating the mixture for 20 min at 37°C. RNA was extracted with phenol-chloroform and precipitated with ethanol. It was then hybridized with the kinase ^{32}P-labeled primer CGCCAAGCTATT TAGGTGACACTAT (5′ end labeled; 1×10^6 to 2×10^6 cpm) in 20 μl of hybridization buffer (10 mM Tris-HCl [pH 7.5], 250 mM KCl, 1 mM EDTA) for 90 min at 37°C. The primer extension reaction was started by adding 40 μl of extension reaction solution (75 mM Tris-HCl [pH 8.0], 15 mM DTT, 12 mM $MgCl_2$, 75 μg of actinomycin D per ml, 12 U of recombinant RNasin, 750 μM each dATP, dTTP, dCTP, and dGTP, 100 U of SuperScript RNase H reverse transcriptase), and the reaction mixture was incubated for 90 min at 37°C. The cDNA products were extracted with phenol-chloroform; precipitated with ethanol; dissolved, and denatured (100°C for 3 min) in 10 μl of 95% formamide containing 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF; and finally analyzed on a 6% polyacrylamide–7 M urea gel.

Protein-protein interaction assay. Recombinant glutathione *S*-transferase (GST) fusion (expressed in bacterial cells) or FLAG-tagged (expressed in insect cells) proteins (1 μg) were immobilized on 10 μl of glutathione or M2 agarose beads, respectively. Then 10 μl of beads was incubated for 2 h at 4°C with 5 μl of rabbit reticulocyte lysate containing [^{35}S]Met-labeled proteins or 100 μl of HeLa nuclear extract (60 μg proteins) in a final volume of 200 μl containing 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 20% glycerol, 2 mM DTT, 150 mM KCl, 0.1% NP-40 and 0.5 mg of BSA per ml. The beads were washed five times (1 ml each) with the incubation buffer, boiled in 10 μl of the 2× SDS gel sample buffer, and analyzed by autoradiography or Western blot analysis. For the coimmunoprecipitation assay, 10 μl of M2 agarose beads was incubated with 250 μl of whole-cell extract from transfected 293T cells in BC150–0.1% NP-40 for 2 h. The beads were washed with the incubation buffer and analyzed by Western blotting.

In situ hybridization. The archival normal prostate tissues were obtained during radical prostatectomy of prostate cancer patients at New York University Medical Center under an Institutional Review Board-approved protocol. The procedure for in situ hybridization was as described previously (29). Briefly, the sections (4 μm) of prostate tissues were hydrated, postfixed in 4% paraformaldehyde, treated with proteinase K, and deacetylated. The prehybridization and hybridization were performed at 68°C. The 536-bp AR (nucleotides 2224 to 2716) and the 648-bp AES (nucleotides 353 to 957) cDNA fragments containing T7 and T3 promoters at each end was generated by PCR. The ^{33}P-labeled probe RNAs (sense and antisense) were generated by in vitro transcription with T7 and T3 RNA polymerases, respectively, and hybridized to the slides containing prostate tissue specimens. After being washed, the slides were exposed for 2 to 3 weeks and then counterstained with hematoxylin and eosin.

RESULTS

N-terminal domain of AR interacts with the Groucho/TLE family protein AES. Various coactivators and corepressors have been shown to play a critical role in mediating the functions of nuclear receptors (56). Although a number of AR-interacting coactivators have been identified (reviewed in references 4 and 19), we have used a yeast two-hybrid screening method to search for additional AR-interacting proteins. For this assay, full-length human AR (residues 2 to 919) was fused to human Sos (hSos) as a bait (Fig. 1A). The temperature-sensitive mutant *S. cerevisiae* strain cdc25H, which contains a point mutation in the yeast homolog (*cdc25*) of the hSos gene, cannot grow at 37°C but can grow at the permissive temperature (25°C). This yeast strain was used to screen a human prostate cDNA expression library fused to the v-Src myristylation sequence, which anchors the fusion protein to the plasma membrane. If the bait and target proteins physically interact, the hSos protein is recruited to the membrane, thereby activating the Ras signaling pathway and allowing the cdc25H yeast strain to grow at 37°C.

Approximately 2 million transformants from the prostate cDNA library were screened, and 35 positive clones were obtained. Nucleotide sequence determination and comparison with GenBank databases (National Center for Biotechnology Information) revealed seven clones that encoded the human AES (5, 11). To confirm that the interactions between AR and AES are specific, human AES was fused to the Gal4 DNA-

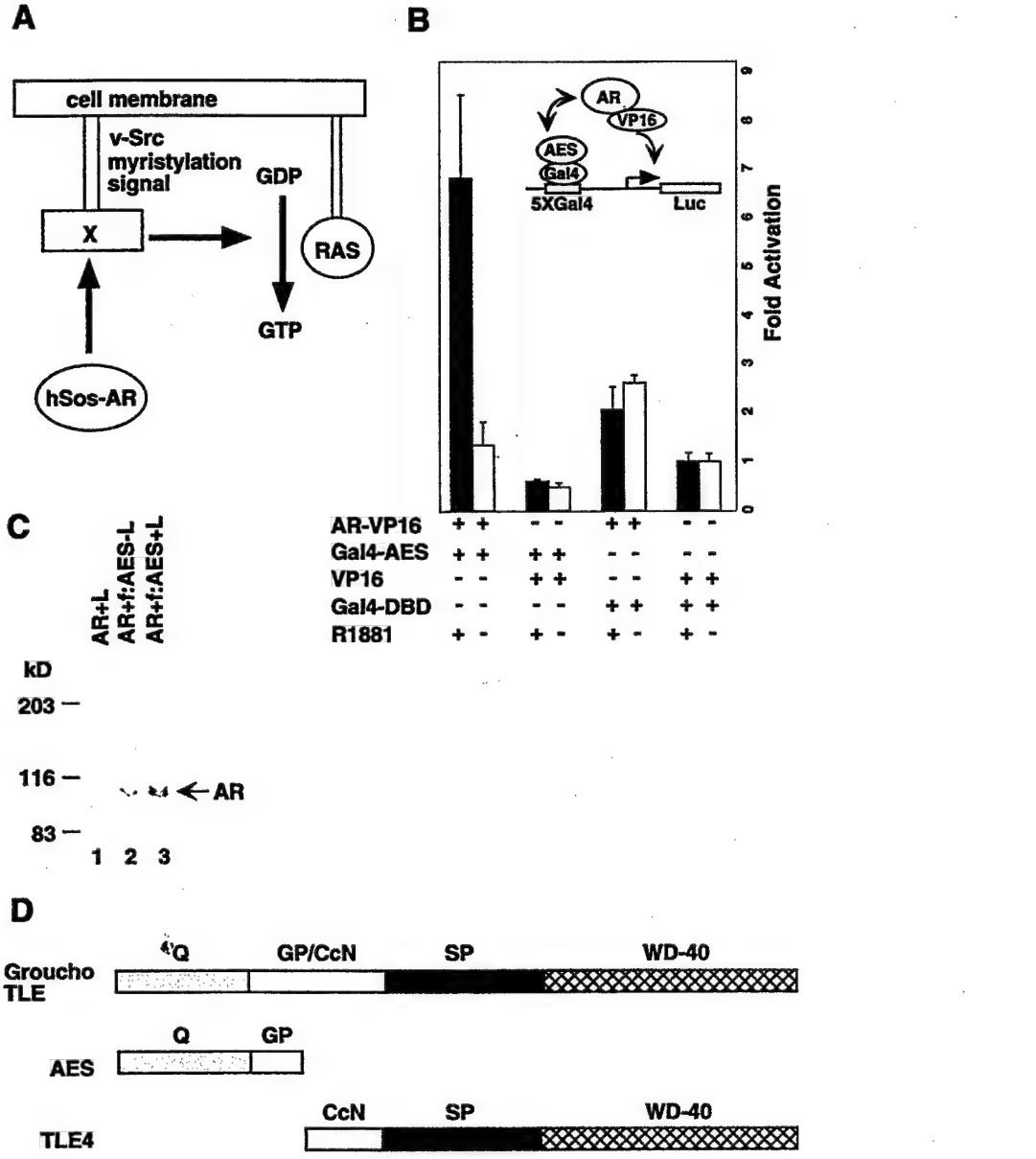

FIG. 1. AR interacts with AES in vivo. (A) Schematic diagram of the Ras signaling pathway utilized in the yeast two-hybrid system. (B) Mammalian two-hybrid assay with Ga14-AES and AR-VP16 fusion proteins in 293T cells. 293T cells were cotransfected with 1 μg of either AR-VP16, Ga14-AES, VP16, or Ga14-DBD in the presence or absence of R1881 (100 nM), along with 100 ng of pG5-Luc reporter plasmid. A significant interaction was detected only between AR and AES. (C) AR was coimmunoprecipitated with AES. 293T cells were transfected with AR (lane 1) or AR and FLAG-tagged AES (lanes 2 and 3) in the presence (lanes 1 and 3) or absence (lane 2) of R1881 (100 nM). Whole-cell extracts were made from the transfected cells and incubated with M2 agarose beads. The immunoprecipitated proteins were analyzed by Western blotting with an anti-AR antibody. (D) Domain structures of three forms of the Groucho/TLE family proteins.

binding domain and AR was fused to the VP16 transcriptional activation domain. These constructs were transfected into 293T cells with a reporter containing five Gal4-binding sites and the E1b core promoter fused to the luciferase gene, and activation of luciferase reporter was measured in the absence and presence of ligand (R1881). A sevenfold activation of the reporter gene was observed in the presence of androgen but not in its absence, indicating that AR-AES interactions are hormone-dependent in vivo (Fig. 1B). As negative controls, neither coexpression of AR-VP16 with Gal4-DBD nor coexpression of Gal4-AES with VP16 resulted in significant ligand-dependent activation of the reporter (Fig. 1B). To further confirm the interaction of AR with AES in mammalian cells, we performed a coimmunoprecipitation using immobilized anti-FLAG monoclonal antibody (M2 agarose). AR was coimmunoprecipitated with AES from the whole-cell extract made from cells transfected with AR and FLAG-tagged AES in the presence of 100 nM ligand (R1881) (Fig. 1C, lane 3). In the absence of ligand, only trace amounts of AR were coimmunoprecipitated (lane 2). As a negative control, no AR was immu-

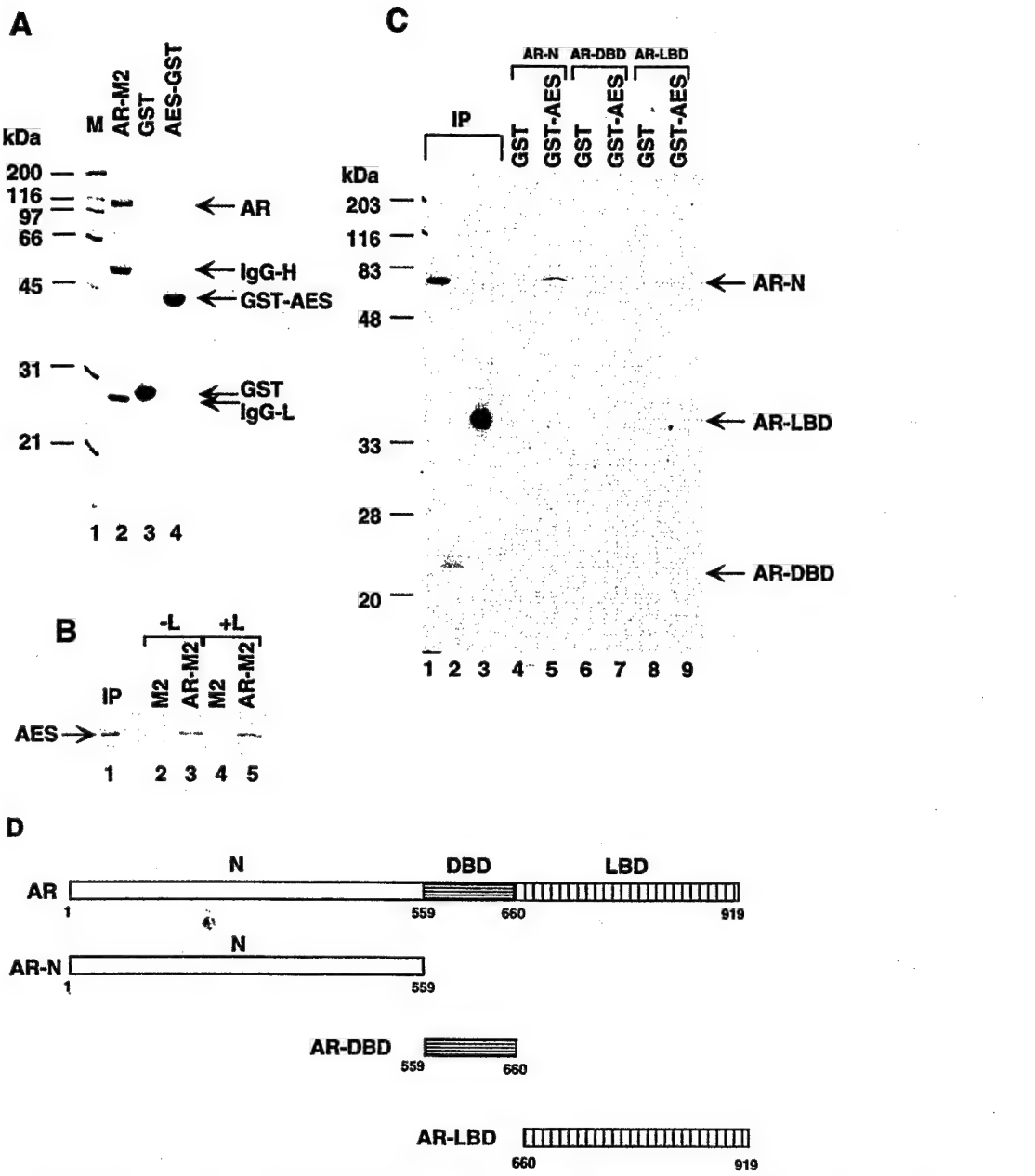

FIG. 2. The N-terminal part of AR directly interacts with AES in vitro. (A) SDS-PAGE (12% polyacrylamide) analysis of the M2 agarose-bound recombinant AR (lane 2), bacterially expressed and purified GST (lane 3), and GST-AES (lane 4) proteins. Standard molecular mass markers (M) (in kilodaltons) are shown in lane 1. IgG light (IgG-L) and heavy (IgG-H) chains of monoclonal antibody (M2) that dissociated from agarose beads by boiling with SDS sample buffer are indicated on the right. (B) AR interacts with AES in vitro independently of the ligand. Radiolabeled AES was incubated with M2 (lanes 2 and 4) or FLAG-tagged AR immobilized on M2 agarose beads (lanes 3 and 5) in the absence (lane 2 and 3) or presence (lanes 4 and 5) of 100 nM R1881. After the beads were washed, bound AES and 5% of the input (IP) (lane 1) were analyzed on by SDS-PAGE (12% polyacrylamide) and visualized by autoradiography. (C) The N-terminal part of AR is sufficient to bind to AES. GST (lanes 4, 6, and 8) or GST-AES (lanes 5, 7, and 9) proteins, immobilized on beads, were mixed with 5 μl of in vitro labeled N-terminal (AR-N) (lanes 4 and 5), DNA-binding (AR-DBD) (lanes 6 and 7), and ligand-binding (AR LBD) (lanes 8 and 9) domains of AR. After the beads were washed, the bound proteins and 5% of the input (IP) (lanes 1 to 3) were analyzed by SDS-PAGE (12% polyacrylamide) and visualized by autoradiography. (D) Diagram of AR, AR DNA-binding domain, and AR ligand-binding domain.

noprecipitated by M2 agarose when the cell was transfected with AR alone (lane 1).

To further investigate the interactions of AES with AR, we performed in vitro protein-protein pull-down assays. In vitro-translated [^{35}S]AES was incubated with FLAG-tagged AR that had been expressed in Sf9 cells and immobilized on M2 agarose beads (Fig. 2A, lane 2). Figure 2B shows that AES bound to AR-M2 (lanes 3 and 5) but not to unliganded M2 agarose beads (lanes 2 and 4). These interactions were found to be ligand independent (Fig. 2B, compare lane 5 with lane 3), a

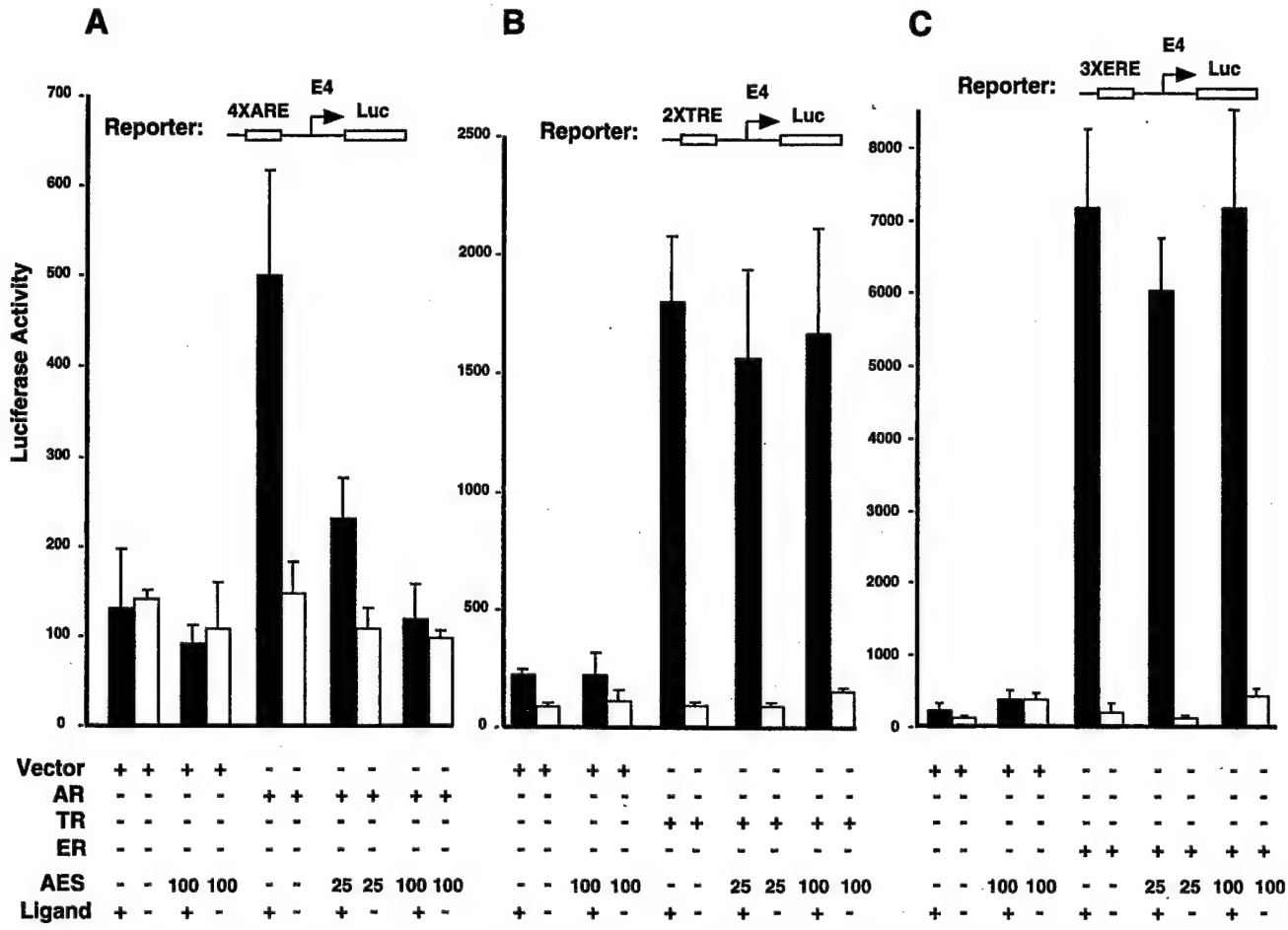

FIG. 3. AES inhibits AR-mediated transcription in vivo. (A) AES represses AR-dependent luciferase gene expression induced by AR in the presence of R1881. HeLa cells were transfected with 500 ng of 4×ARE-E4-luc reporter plasmid, 30 ng of pCMV-AR, and the indicated amounts of pCMV-AES expression plasmids. Cells were grown in the absence or presence of 100 nM R1881 for 48 h after transfection and then harvested for luciferase activity assays. (B) TR-mediated T3-dependent luciferase gene expression is not suppressed by AES. The reporter construct contains 2×TRE, the E4 core promoter, and the luciferase gene. After transfection, HeLa cells were grown for 48 h in the absence or presence of 10 nM T3 before being harvested for luciferase assays. (C) ER-mediated estradiol-dependent luciferase gene expression is not suppressed by AES. The reporter construct contains 3×ERE, the E4 core promoter, and the luciferase gene. After transfection, HeLa cells were grown for 48 h in the absence or presence of 1 μM estradiol before the luciferase assays were performed.

somewhat surprising observation in view of the observed ligand-dependent interactions in vivo (Fig. 1B). This discrepancy is probably because AR associates with heat shock proteins and other chaperones in vivo in the absence of androgen (30), thereby preventing its interactions with AES as well as other cofactors. To identify the AR domain that interacts with AES, the N-terminal, DNA-binding, and ligand-binding domains of AR (Fig. 2D) were expressed as ^{35}S-labeled proteins and incubated with GST and GST-AES fusion protein immobilized on glutathione-agarose beads (Fig. 2A, lanes 3 and 4). As shown in Fig. 2C, the N-terminal part bound to GST-AES (lane 5) but not to GST alone (lane 4) whereas the DNA-binding and ligand-binding domains failed to interact (lanes 7 and 9). This demonstration that AES interacts with the AR N-terminal region is interesting in light of the significant role of this region in target gene activation by liganded AR (26).

AES represses AR-dependent gene expression. We then investigated the effect of AES on AR-dependent transcription by performing transient-transfection assays. The luciferase reporter plasmid containing four tandem copies of the PSA gene androgen-responsive elements (7) upstream of the minimal adenovirus E4 promoter (see Fig. 5C) was cotransfected with expression vectors for AR and/or AES into HeLa Z cells in the absence or presence of ligand (R1881). As shown in Fig. 3A, AR activated the reporter gene about fourfold in the presence of androgen, and coexpressed AES completely blocked this AR-dependent transactivation in a dose-dependent manner. In the absence of cotransfected AR or ligand (R1881), AES did not influence reporter gene activity, indicating that the inhibitory effect of AES on AR-dependent gene expression was not due to an effect on the E4 promoter. Similar results were obtained with the LNCaP prostate cancer cell line (data not shown).

To further examine whether the inhibitory effect of AES is specific for AR, we compared the effects of AES on the transcription of reporters containing the same E4 promoter under the control of TR and ER. As shown in Fig. 3B and C, TR and ER activated the reporter genes about 17- and 35-fold, respec-

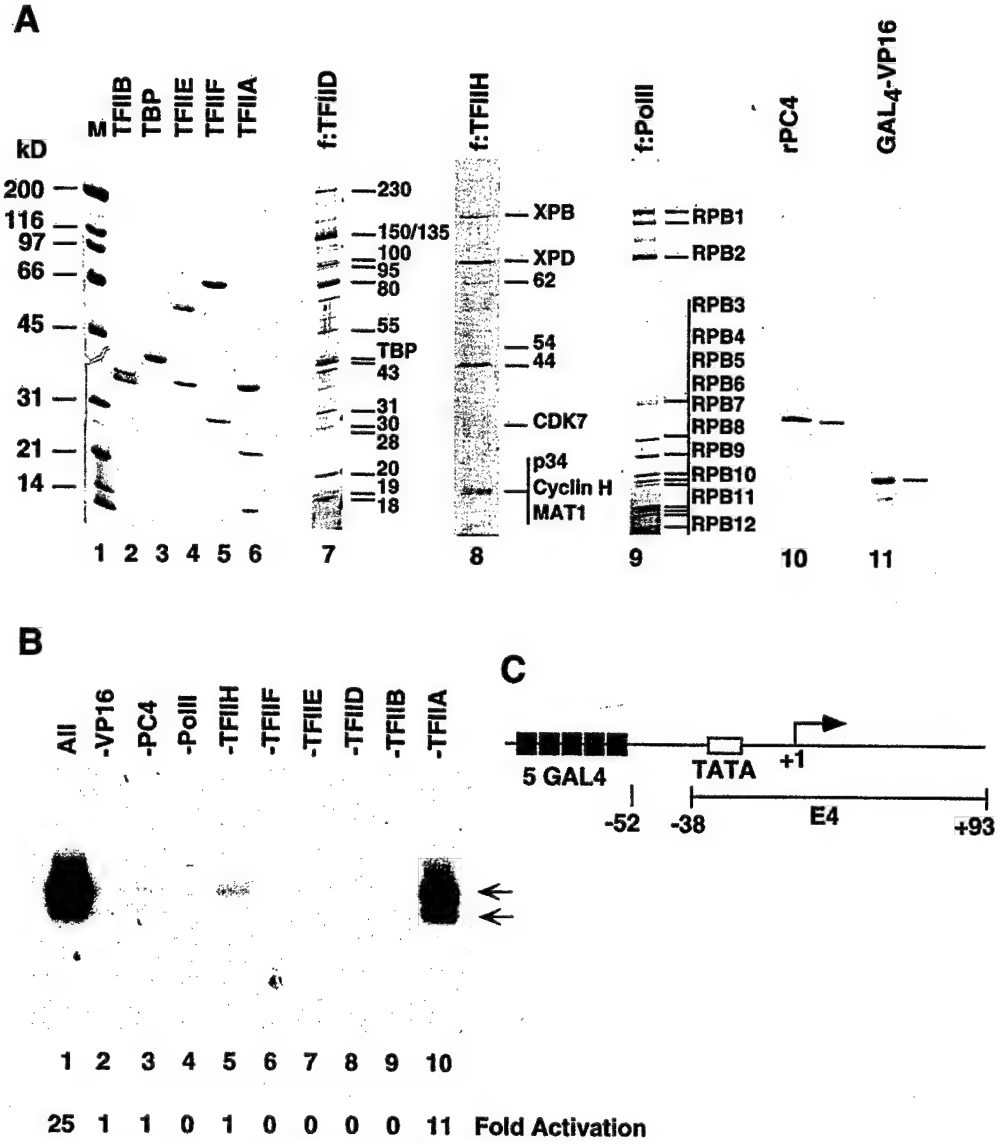

FIG. 4. Transcription activation by a model activator in a cell-free system reconstituted with purified factors. (A) SDS-PAGE analysis of purified factors. Coomassie blue R250 staining of purified recombinant activator GAL4-VP16 (lane 11), the general coactivator PC4 (lane 10), and the general initiation factors TFIIA, TFIIB, TFIIE, TFIIF, and TBP (lanes 2 to 6) was performed. Silver staining of the immunopurified FLAG-tagged multisubunit general initiation factors TFIID and TFIIH and RNA polymerase II (lanes 7 to 9) was performed. The subunits identified as integral subunits are indicated by size (in kilodaltons) or by name on the right. Some bands are difficult to visualize because of weak or negative staining. Unmarked bands represent either degradation products or contaminants that can be removed by further purification. Lane 1 shows molecular weight markers (M), (B) Activator-dependent transcription. Transcription was conducted with the purified components shown in panel A and the DNA template indicated in panel C. The two arrows show specifically initiated transcripts assayed by primer extension. A complete reaction with all factors is shown in lane 1, whereas reactions with single-factor omissions (indicated at the top) are shown in lanes 2 to 10. Fold activation above the basal level (-GAL4-VP16, lane 2) is indicated at the bottom. (C) Diagram of the model template. The template contains five tandem GAL4 sites adjacent to the adenovirus E4 core promoter.

tively, in the presence of their cognate ligands (T3 and estradiol). In contrast to its dramatic effect on AR-mediated transactivation, AES showed no effect on TR- or ER-mediated transcription. Western blot analysis revealed that the expression levels of ER were comparable to those of AR (data not shown). Hence, AES shows nuclear receptor-specific inhibitory effects in vivo.

Establishment of a highly purified in vitro transcription system for activator function. To study the mechanism of basal and activator-dependent transcription, we established an activator-responsive complementation assay involving homogenous recombinant and FLAG-tagged immunopurified natural general initiation factors (TFIIs) and positive cofactors (PCs) (43, 44). The recombinant factors expressed in and purified from bacteria included TFIIA (three subunits [Fig. 4A, lane 6]), TFIIB (one subunit [lane 2]), TFIIE (two subunits [lane 4]), TFIIF (two subunits [lane 5]), and PC4 (1 subunit [lane 10]). The multisubunit components purified from cell lines

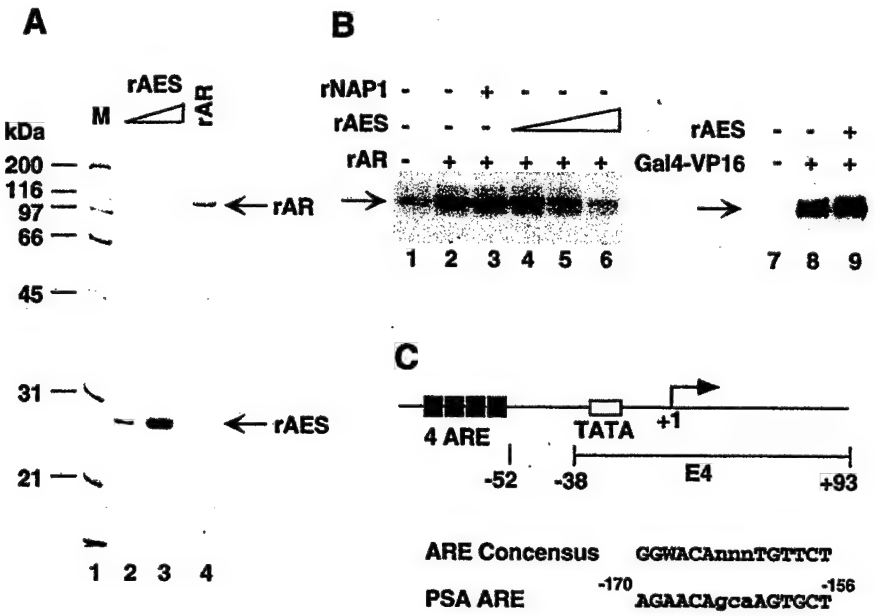

FIG. 5. AES represses AR-driven transcription in vitro. (A) SDS-PAGE analysis of recombinant AES and AR proteins. Portions of 50 (lane 2) and 200 (lane 3) ng of purified recombinant 6His-tagged AES expressed in bacteria and 100 ng of purified recombinant human AR (lane 4) expressed in Sf9 cells were subjected to SDS-PAGE with Coomassie blue R250 staining. (B) AES inhibition of AR-dependent transcription. A synthetic template containing four ARE elements (pARE-E4) was transcribed in a system reconstituted with the purified factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, Pol II, and PC4) as shown in Fig. 1, panel A, and 10 ng of affinity-purified (via f:Nut2) TRAP/Mediator complex (27). Other additions included 30 ng of rAR (lanes 2 to 6); 10 (lane 4), 30 (lane 5) or 100 (lanes 6 and 9) ng of rAES; 100 ng of rNAP1 (lane 3); and 30 ng of Gal4-VP16 (lanes 8 and 9). The specifically initiated transcript is indicated by an arrow and was monitored by primer extension. (C) Diagram of the synthetic ARE-containing promoter. The template (pARE-E4) contains four tandem copies of the ARE from the PSA promoter positioned upstream of the adenovirus E4 promoter.

expressing FLAG-tagged subunits included f:TFIID (~15 subunits [Fig. 4A, lane 7]), f:TFIIH (9 subunits [lane 8]), and f:Pol II (12 subunits [lane 9]). Recombinant GAL4-VP16 (lane 11) was used as an activator to establish the functionality of this particular assay system. The GAL4-VP16-responsive template pG5E4 (Fig. 4C) contains five Gal4-binding sites preceding the adenovirus E4 core promoter (from −38 to +93) (36). To determine whether all purified factors are required for transcription in our highly purified transcription system, we first tested a complete mixture of all GTFs, Pol II, Gal4-VP16, and PC4 with supercoiled DNA template (pG5E4) and then omitted individual factors. As shown in Fig. 4B, basal (activator-independent) transcription (lane 2) is completely dependent on TFIID (lane 8), TFIIB (lane 9), TFIIE (lane 7), TFIIF (lane 6), and Pol II (lane 4) whereas activation (up to 25-fold) by GAL4-VP16 (lane 1) absolutely requires Pol II and all initiation factors other than TFIIA.

AES represses AR-driven transcription in vitro. For initial tests of AR function, we constructed a synthetic hybrid promoter (pARE-E4) containing four copies of an androgen response element (ARE) from the PSA promoter (7, 42) just upstream of the adenovirus E4 core promoter (Fig. 5C). This template was assayed in the above-described purified system supplemented with an affinity-purified TRAP complex previously shown to facilitate transcription by other nuclear receptors (12, 17, 58). In this system, the purified baculovirus-expressed recombinant AR (Fig. 5A, lane 4) activated transcription threefold (Fig. 4B, compare lane 2 with lane 1). To test its effect on AR-dependent transcription in this system, human AES was expressed in and purified from bacteria (Fig. 5A, lanes 1 and 2). Addition of recombinant AES inhibited AR-dependent transcription in a dose-dependent manner and, at the highest level (100 ng), reduced it to the basal level (compare lane 6 and lane 1). As negative controls, 100 ng of recombinant mouse NAP1 expressed and purified in a manner identical to that for AES had no detectable effect on the AR-driven transcription (compare lane 3 and lane 2) and recombinant AES did not repress but slightly (1.5-fold) enhanced Gal4-VP16-driven transactivation (compare lane 9 and lane 8) in the same reconstituted system. These results suggest that AES specifically and directly represses AR-driven transcription in vitro.

AES interacts with TFIIE. The structural resemblance of Groucho/TLE proteins to Tup1 (21), a general transcription repressor in yeast, suggested that the two proteins may function by a similar mechanism. More recently, Gromoller and Lehming (14) reported TUP1-mediated repression through physical interaction with the SRB7 subunit of the yeast Mediator complex. To test whether human AES uses a similar mechanism to repress transcription, we performed protein-protein pull-down assays. GST-AES failed to bind the human homolog (TRAP-Mediator complex, detected by anti-TRAP95 antibodies) of the yeast Mediator complex (27) in HeLa nuclear extract (Fig. 6A), as well as the independently expressed SRB7 and MED7 subunits of the human TRAP-Mediator complex (Fig. 6B, lanes 4 and 6). These results suggest that human AES, unlike yeast Tup1, may not directly interact with the Mediator complex.

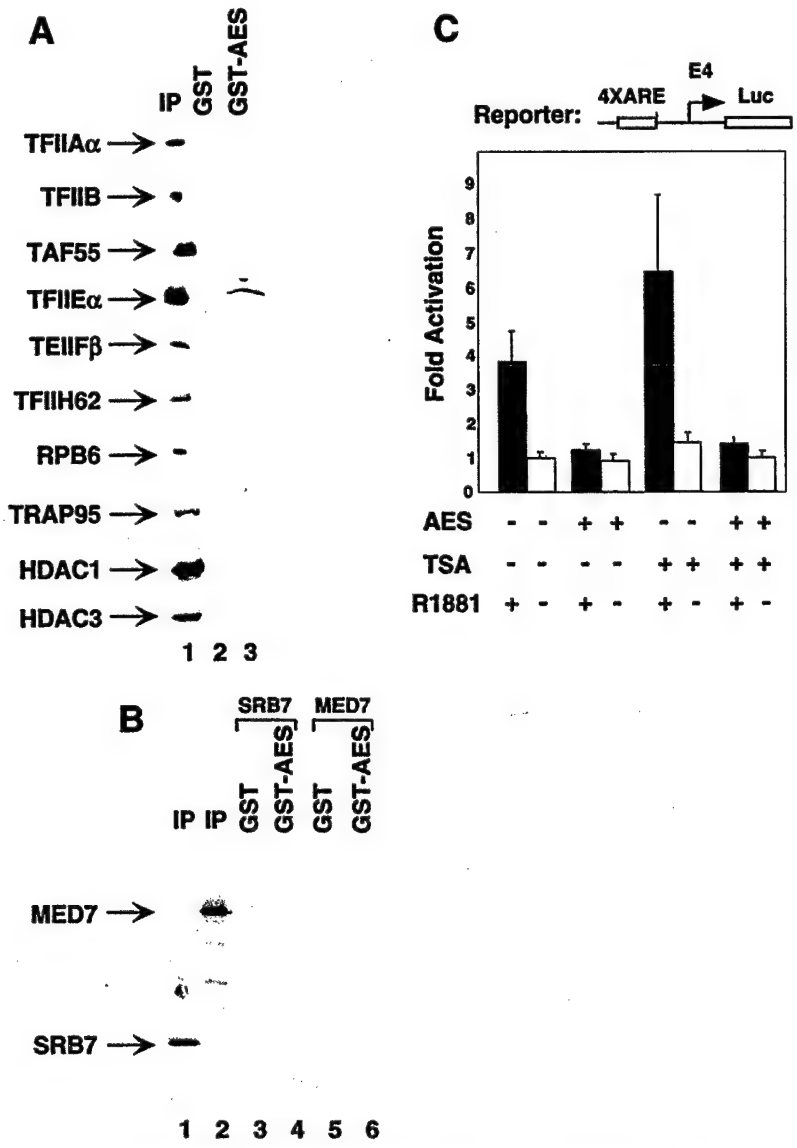

FIG. 6. AES interacts with TFIIE and represses AR-dependent transcription in the presence of TSA. (A) GST (lane 2) and GST-AES (lane 3), immobilized on agarose beads, were incubated with HeLa nuclear extract. The bound proteins (lanes 2 and 3) and 10% of the input (IP) were analyzed by Western blot assays with the corresponding antibodies indicated on the left. (B) AES does not interact with MED7 and SRB7. GST (lanes 3 and 5) or GST-AES (lanes 4 and 6) proteins, immobilized on beads, were mixed with 5 μl of in vitro-labeled SRB7 (lanes 3 and 4) and MED7 (lanes 5 and 6). After the beads were washed, the bound proteins and 10% of the inputs (IP) (lanes 1 and 2) were analyzed by SDS-PAGE (15% polyacrylamide) and visualized by autoradiography. (C) AES inhibits AR-dependent transcription in the presence of TSA. HeLa cells were transfected with 100 ng of 4×ARE-E4-luc reporter plasmid, 50 ng of pCMV-AR, and 100 ng of pCMV-AES expression plasmid. Cells were grown in the absence or presence of 100 nM R1881 and 10 ng of TSA per ml for 48 h after transfection and then were harvested for luciferase activity assays.

HDAC-containing complexes mediate the function of various corepressors in vivo (15, 35). To investigate whether AES also functions through these complexes, we performed transient-transfection assays in the presence of the general deacetylase inhibitor TSA. Figure 6C shows that AES still actively inhibits AR-dependent transcription in the presence of TSA and at a level similar to that observed in the absence of TSA. These results suggest that AES represses AR-dependent transcription by directly targeting the basal transcriptional machinery rather than through chromatin modifications involving recruitment of HDAC-containing corepressor complexes. This conclusion is further supported by the absence of demonstrable interactions of AES with HDAC1- and HDAC3-containing complexes (16, 25) in HeLa nuclear extract (Fig. 6A). However, since the reporter gene in the transient-transfection assay may not be packaged appropriately into chromatin, we cannot rule out the possibility of the involvement of HDACs in AES function on endogenous genes within chromatin.

To further study the mechanism of action of AES, we performed additional protein-protein pull-down assays to assess possible interactions of AES with components of the basal transcriptional machinery. GST and GST-AES agarose beads were incubated with HeLa nuclear extract, and the bound proteins were analyzed by Western blot assays with polyclonal

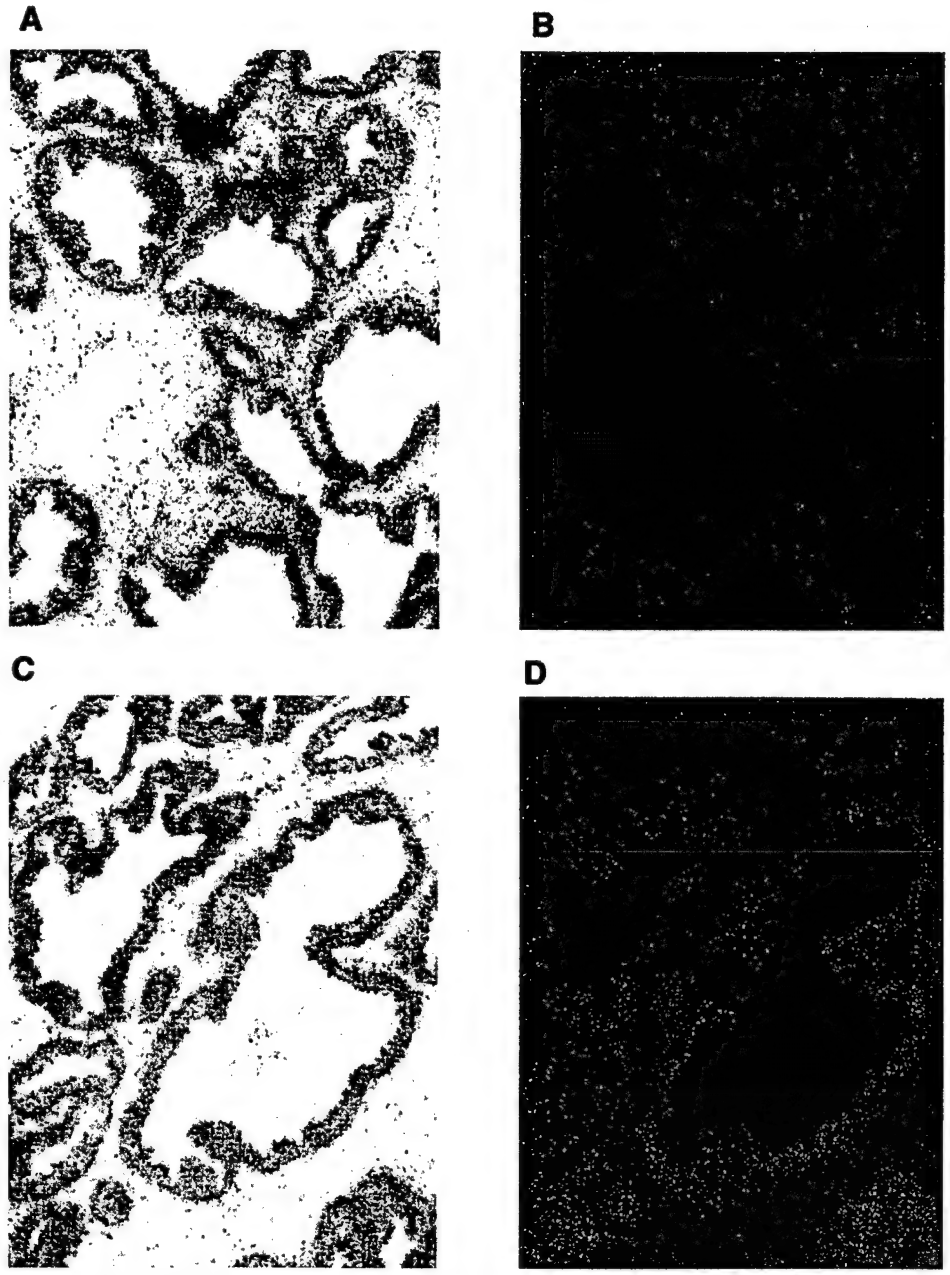

FIG. 7. AES expressed in the epithelial cells of the prostate. The slides containing sections of prostate tissues were hybridized with antisense AR (A and B) or AES (C and D) RNA probes. The emulsion-coated slides were exposed and evaluated under a Nikon microscope with a digital camera interfaced to a computer. The left and right panels show bright-field and dark-field images of the same area of the slides.

antibodies against subunits of RNA polymerase II and basal transcription factors. As shown in Fig. 6A, TFIIE (detected by antibodies against TFIIEα) was specifically retained by GST-AES, relative to GST alone, whereas other basal transcription factors (TFIIA, TFIIB, TFIID, TFIIF, and TFIIH) and RNA polymerase II failed to be bound. These observations implicate TFIIE as a possible target for AES.

AES is highly expressed, along with AR, in prostate epithelial cells. To determine whether AES and AR are expressed in the same cells in humans, we investigated the expression levels of AES and AR in normal prostate tissues by in situ RNA hybridization. Consistent with previous observations (32), the expression levels of AR were high in the epithelial cells of the prostate (Fig. 7A and B). Expression of AES was also evident in the epithelial cells (Fig. 7C and D). As negative controls, no hybridization signals above the background levels were detected with the sense RNA probes, thus indicating that the signals obtained with the antisense probe are specific (data not shown).

DISCUSSION

Various cofactors that have been implicated in the function of AR, as well as a number of other nuclear receptors, include p300/CBP, p160 family proteins, the ARA group (ARA24,

ARA45, ARA54, ARA55, ARA70, and ARA160), ARIP3, SNURF, and BAG-1L (18, 19, 46). All of these enhance AR-mediated transcription in vivo, although there is not a clear mechanistic understanding of the function of these factors. The results described here demonstrate (i) that AES is a selective repressor of ligand-dependent AR-mediated transcription and (ii) that AES physically interacts with the N-terminal region of AR and represses AR-driven transcription by targeting the basal transcriptional machinery (possibly TFIIE). These observations thus reveal a new negative regulatory pathway for AR function, as well as new insights into the mechanism of action of mammalian Groucho/TLE proteins.

AES represses AR-dependent transcription. A number of proteins have been demonstrated to repress AR-dependent transcription in vivo. These include AP-1 (34, 47), NF-κB (39), TR4 (testicular orphan receptor 4) (24) and HBO1 (histone acetyl transferase binding to origin recognition complex 1) (49). AP-1, NF-κB, and TR4 appear to inhibit AR-dependent transcription by mutual transcriptional interference (unexpected interactions of distinct transcription factors). Although the molecular mechanisms that underlie this phenomenon have remained mostly elusive, this may involve competition for a coactivator commonly required by both activators (1).

HBO1 belongs to the MYST family, which is characterized by highly conserved C_2HC zinc fingers and a putative histone acetyltransferase domain. HBO1 contains a putative repression domain, interacts with the DBD-LBD of AR, and inhibits AR-dependent transcription in vivo, although the exact mechanism of HBO1 action remains to be determined (49). Based on the results presented here, AES represses AR-driven transcription in a manner more like that of HBO1. Like HBO1, AES physically interacts with AR and specifically represses AR-dependent transcription in transient-transfection assays. Also as reported for HBO1, AES probably does not act broadly as a nuclear hormone receptor corepressor because it represses AR-dependent transcription but not TR- or ER-dependent transcription. These results are consistent with the fact that AES physically interacts with the N-terminal region of AR, which is not conserved in the N-terminal regions of TR and ER (37). Nonetheless, it remains important to determine whether AES might repress other (as yet untested) nuclear receptors and, related, whether other members of the Groucho/TLE family can repress the function of AR or other nuclear receptors.

Like AR, some of the DNA-binding partners for the Groucho/TLE proteins do not always act as transcriptional repressors, and, in fact, some are better characterized as activators (11). For the Groucho-interacting Dorsal and Runt domain proteins (2, 9, 20, 22), the context of the target gene promoter appears to be critical for determining whether activation or repression will occur. These observations suggest that the recruitment of Groucho/TLE proteins and/or their repressor activities might also be dependent on the nature of the target gene promoter. It is also possible that Groucho/TLE proteins might function as coactivators in certain situations. Thus, it will be important to determine whether AES repression of AR-driven transcription is dependent on the target gene promoter context.

Mechanism of AES function. At present, relatively little is know about the mechanisms by which Groucho/TLE family proteins function as eukaryotic (co)repressors. Various repressors and activators recruit the Groucho/TLE proteins through specific interactions with various regions of Groucho proteins (11). In the well-defined reconstituted transcription system utilized here, we observed repression of AR-dependent transcription from DNA templates by recombinant AES. Consistent with the indication from this result that AES may function through interactions with the basal transcriptional machinery, a specific interaction of AES with the basal transcription factor TFIIE was observed. Similarly, previous studies have shown that the zinc finger protein Kruppel represses transcription through physical interactions with TFIIE (48). Hence, these studies suggest that TFIIE may serve as a more general target for various corepressors and repressors.

TUP1, a general transcriptional corepressor (21, 50), is a yeast analog of the Groucho/TLE proteins. Gromoller and Lehming (14) demonstrated that the essential holoenzyme component SRB7 is a physical and functional target of TUP1. In addition, genetic interactions between Cyc8-Tup1 and a variety of Pol II holoenzyme components (SRB8, SRB10, SRB11, Sin4, Rgr1, Rox3, and Hrs1) have been reported (23). However, we failed to detect direct interactions of AES with human SRB7 or the SRB7-containing TRAP/Mediator complex in protein-protein pull-down assays, indicating that human AES may not directly target human SRB7 or the TRAP/Mediator complex. This observation may reflect the fact that Tup1 and Groucho/TLE proteins show poor sequence conservation (at the amino acid level) in both repression domains and WD-40 repeats. Similar to AES and suggesting a chromatin-independent mechanism, the purified Tup1-containing complex directly represses transcription in a crude yeast extract in vitro (40).

Many corepressor complexes contain HDAC enzymes. The *Drosophila* HDAC Rpd3 has been identified as a Groucho-interacting protein (6), and, possibly related, Groucho proteins also interact with histone H3 (38). Yeast Tup1 similarly interacts directly and genetically with histones H3 and H4 (10, 38), and mutations in genes encoding the HDACs abolish Tup1-mediated repression (55). These findings have led to a repression model, possibly complementing the more direct mechanisms indicated above, involving Groucho/Tup1 recruitment by promoter-bound factors, HDAC recruitment by Groucho/Tup1, and subsequent function of HDAC to establish and/or maintain a transcriptionally silenced chromatin structure. Our results do not support this model for AES. First, we failed to detect interactions between AES and HDAC1- or HDAC3-containing complexes. Second, the deacetylase inhibitor TSA did not affect AES-mediated inhibition of AR-dependent transcription in transient-transfection assays. Third, we observed a direct inhibition of AR-dependent transcription by recombinant AES in a highly purified reconstituted transcription system on a naked DNA template.

In summary, our results point both to a novel function for AES in mediating repression of AR-dependent transcription and to a mechanism involving direct interactions both with AR and with the basal transcription machinery. AR is an important regulatory factor in the development, differentiation, and maintenance of male reproductive functions, as well as in the regulation of other sexually dimorphic processes ranging from the development of neural tissues to the modulation of im-

mune function (33). Thus, the mammalian Groucho-related protein AES, and possible other family members, may play a pivotal role in these biological processes by modulating the transcriptional activity of AR.

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Heterogeneous Expression and Functions of Androgen Receptor Cofactors in Primary Prostate Cancer

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Abstract

The androgen receptor (AR), a ligand-activated transcription factor of the steroid receptor superfamily, plays an important role in normal prostate growth and in prostate cancer. The recent identification of various AR cofactors prompted us to evaluate their possible roles in prostate tumorigenesis. To this end, we analyzed the expression of AR and eight of its cofactors by quantitative in situ RNA hybridization in 43 primary prostate cancers with different degrees of differentiation. Our results revealed nearly constant expression of AR and heterogeneous expression of AR cofactors, with increased expression of PIAS1 and Ran/ARA24, decreased expression of ELE1/ARA70, and no change in TMF1/ARA160, ARA54, SRC1, or TRAP220. Interestingly, whereas TMF1/ARA160, ELE1/ARA70, ARA54, RAN/ARA24, and PIAS1 were preferentially expressed in epithelial cells, another cofactor, ARA55, was preferentially expressed in stromal cells. Though the changes in levels of these coactivators did not correlate with Gleason score, their occurrence in high grade prostatic intraepithelial neoplasia, suggests their involvement in initiation (or an early stage) of cancer. In addition, human prostate tumor cell proliferation and colony formation were markedly reduced by ELE1/ATRA70. Together, these findings indicate that changes in levels of expression of AR cofactors may play important, yet different, roles in prostate tumorigenesis.

Introduction

Androgens mediate development and maintenance of normal prostate tissue and also seem to be involved in prostate tumor growth and progression.[1] Androgens act through the androgen receptor (AR), which belongs to the large family of nuclear receptors.[2] These receptors are hormone-activated transcription factors and structurally conserved. Activation of AR by androgens is a multistep process that involves androgen binding to the receptor, an accompanying structural change in the receptor, loss of associated heat shock/chaperone proteins, translocation of the liganded receptor to the nucleus, and binding of the liganded receptor to target genes. There is increasing evidence that the transcriptional activity of AR and other nuclear receptors depends on their interaction with various cofactors (coactivators and corepressors).[3,4] A variety of cofactors have been identified by their ability to bind various nuclear receptor domains and to alter the transcriptional activity of nuclear receptors following overexpression in cell lines.[5] The best-studied group includes p300/CBP, the p160 family (SRC-1, TIF-2/GRIP-1, ACTR/P-CIP),[5] and PCAF/GCN5 complexes (yeast SAGA, human STAGA).[6,7] All have histone acetyltransferase (HAT) activities and are thought to act mainly through histone acetylation and consequent chromatin structural perturbations, although they can also act through functional acetylation of activators[8] and coactivators.[9] A second group includes the TRAP components of the TRAP/DRIP/ARC/SMCC/Mediator complex,[10] which shows subunit-specific interactions with both

nuclear receptors (mainly through TRAP220) and other activators.[10] This complex in turn facilitates the function of RNA polymerase II and the general initiation factors on DNA templates at post-chromatin-remodeling steps.[10,11] Of these various coactivators, p300/CBP[12,14] and p160s[12,14-16] have been shown to function with AR. Other cofactors implicated in the function of AR and, in most cases, other nuclear receptors, include the ARA group (ARA24, -54, -55, -70 and -160)[17-20], ARIP3[21], SNURF[22] and AES[23].

Altered expression of nuclear hormone receptor cofactors has been implicated in the genesis and progression of breast cancers. Increased expression of TIF2, CBP, and steroid receptor RNA activator has been observed in breast tumor tissues.[24-26] Peroxisome proliferator-activated receptor binding protein (PBP/TRAP220) and SRC3/AIB1 genes are frequently amplified and overexpressed in breast tumors.[27,28] In comparison, however, little is known about the possibility of abnormal expression of cofactors in prostate cancer. Recently, Fujimoto et al.[49] found that the expression levels of ARA55 and SRC1 were higher in cancer specimens with a poor response to endocrine therapy than in those with a good response to endocrine therapy. To explore this question, we analyzed the levels of expression of both relatively AR-selective cofactors (TMF1/ARA160, ELE1/ARA70, ARA55, ARA54, Ran/ARA24, and PIAS1) and more general cofactors (SRC1, TRAP220) in human prostate cancer tissue by quantitative in situ hybridization. Among the tested cofactors, PIAS1 and RAN/ARA24 showed significantly higher expression levels in cancer tissue compared with benign. In contrast, expression of ELE1/ARA70 was

dramatically decreased in primary prostate tumor tissues. A subsequent analysis has demonstrated suppression of LNCaP cell growth by ELE1/ARA70. Collectively, these results imply that these cofactors likely play important but contrasting roles in prostate cancer differentiation and tumorigenesis.

Materials and Methods

Prostate Tissue Specimens and Pathologic Evaluation

Prostate cancer and normal control tissues were derived from radical prostatectomy specimens of 43 prostate cancer patients treated at New York University Medical Center. The study protocol was approved by Institutional Review Board of New York University Medical Center. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of tissue (4 μm) were cut and mounted on Super-frost Plus adhesion slides and used for histology, immunohistochemistry, and in situ hybridization. Prostate cancer foci were categorized as well differentiated (combined Gleason score 2-4)(n = 9), moderately differentiated (combined Gleason score 5-6)(n=17) and poorly differentiated (combined Gleason score 7 and 8-10)(n = 17). The histological features and the Gleason score of each individual specimen were confirmed by two pathologists (J. M. and P.L.).

Immunohistochemistry

The immunohistochemical staining was performed on an automated Ventana machine. Prior to staining, antigen retrieval was performed by heating the specimens in a microwave oven for 30 minutes in citrate buffer (pH 8.0) following dewaxing. A rabbit polyclonal anti-AR antibody (Santa Cruz Biotechnology, Inc.) was applied to the sections at a 1:100 dilution, and sections were then incubated overnight at 4 °C. A streptavidin-biotin peroxidase

detection system was used according to the manufacturer's instruction (DAKO), with 3, 3'-diaminobenzidine as substrate.

In Situ Hybridization

The EST cDNA clones of interest were obtained from Research Genetics, Inc. Oligonucleotides were designed to bear T7 promoter sequences on one end and T3 promoter sequences on the opposite end (Table 1) such that the sense and antisense probes were specified by the polymerase used. Fragments of corresponding genes (approximately 500 bp DNA) were amplified using polymerase chain reaction. High-specific-activity ^{33}P-labeled RNA probes were synthesized by incubation of DNA with T7 or T3 RNA polymerase, 500 μM GTP, ATP and CTP; 3 μM of UTP; and 100 μCi α-^{33}P-UTP (6,000 Ci/mmol) at 37°C for 45 minutes followed by DNase treatment for 15 minutes at 37°C. The probes were purified by chromatography on a Sephadex G-50 column. The yield and quality of the probes were assessed by trichloroacetic acid precipitation and scintillation counting, as well as by agarose gel electrophoresis and autoradiography.

After wax removal and rehydration, 4-μm sections of formalin-fixed tissue were hybridized to the sense and antisense probes following the described procedures[29]. Then, slides were subjected to autoradiography by dipping in NTB-2 X-ray emulsion (Kodak), exposed for 1 to 2 weeks, developed in D-19 developer (Kodak), and fixed in G33C fixer. Lastly, the slides were counterstained with Gills Hematoxylin Stain.

Image and Statistical Analyses

The benign and malignant tissue components were compared on the same section to eliminate tissue-to-tissue and slide-to-slide variations of grain signals. We first examined the consistency of quantification of a given case by analyzing randomly selected areas of non-neoplastic and cancer foci in the cancer specimens for five cases. The results from block to block were comparable in all cases (data not shown). These results validated the in situ hybridization approach with formalin-fixed and paraffin-embedded tissues for analyzing the expression of AR and its cofactors. Slides were evaluated under a microscope (Nikon Eclipse E400) equipped with a digital camera (Princeton Instruments, Inc.) interfaced to a computer with IPLab software. The specimens were categorized into four groups according to degree of differentiation by Gleason score; Gleason score 4, Gleason score 5-6, Gleason score 7, and Gleason score 8-10. The grain (in situ signal) numbers from the areas of interest (cancer, prostatic intraepithelial neoplasia and normal) were captured and counted using IPLab software, and divided by the number of cells to quantify as grain number/cell. An average of 30-50 cells were analyzed for each case. Differences in expression levels of the genes of interest among these four groups were subjected to nonparametric Kruskal-Wallis ANOVA analyses. Results were further grouped according to changes in RNA levels (malignant versus benign tissue) of less than two-fold and greater than two-fold and analyzed by the chi-square test.

Assays of Cell Growth Suppression

LNCaP cells were plated in six-well plates (35-mm wells) and grown in RPIM 1640 medium to about 70% confluency for transfection with plasmid DNA (pcDNA3.1 and pcDNA-ELE/ARA70) and 6 µl of Lipofectamine (Invitrogen Life Technologies). Two days later, cells were selected with G418 at 0.8 µg/ml. After 4 weeks of selection with the medium change every 3 days, the cells were rinsed with PBS, fixed with 2% formaldehyde in PBS for 15 minutes, stained with 0.5% crystal violet in PBS for 15 minutes, rinsed once or twice with distilled water, dried, and stored for subsequent quantification of colonies.

Results

Analysis of mRNA Expression by In Situ Hybridization

A large number of cofactors that regulate AR-driven transcription have been identified.[4] To determine possible relationships to prostate tumorigenesis and prostate cancer progression, we investigated the expression levels of various AR cofactors in normal and tumor prostate tissues by in situ hybridization. In order to validate the in situ hybridization results, we performed both in situ hybridization and immunohistochemical analysis for AR with the same set of slides from the formalin-fixed, paraffin-embedded tissue blocks. Strong and uniform immunostaining of AR

was observed in the nuclei of both epithelial and stromal cells in the benign areas of each of the 43 specimens (Figure 1G). Consistently, AR mRNA expression levels were high in epithelial cells and lower in stromal cells revealed by in situ hybridization (Figure 1A and 1B). As a negative control, only background signals were detected with the sense AR probe (Figure 1C), indicating that the signals obtained with the antisense AR probe were specific.

Expression of AR and Cofactor mRNAs: Epithelial Expression Versus Stromal Expression

In the 43 cases studied, the expression levels and overall expression patterns of AR did not differ significantly (less than two-fold) between normal prostate tissue (from the same section of and adjacent to the prostate cancer region) and prostate cancer tissue. There was no apparent relationship between the amount of AR and the degree of tumor differentiation. These results are in accordance with previous reports that AR is highly expressed in a variety of normal and malignant human prostate tissues.[30] Expression levels of a panel of eight proteins described as modulators of AR function were then analyzed in the 43 prostate tumor samples. Two of these cofactors, SRC1 and TRAP220, interact with a broad spectrum of different nuclear receptors. The other six cofactors studied (the members of the ARA group [TMF1/ARA160, ELE1/ARA70, ARA55, ARA54 and Ran/ARA24] and PIAS1) are relatively specific for AR. The in situ hybridization results are summarized in Table 2. Expression of SRC1 and TRAP220 was detected in both

stromal cells and epithelial cells, whereas expression of ARA54 was observed predominantly in epithelial cells. The expression levels of these mRNAs did not differ significantly between normal and tumor tissues. Although ARA55 mRNA was moderately expressed in stromal cells, it was undetectable in glandular epithelial cells (Figure 1D and E), implying that ARA55 might regulate AR function in prostate stroma. Immunohistochemical staining with anti-ARA55 antibody on the same prostate tissues further confirmed that ARA55 was only expressed in stromal cells (Figure 1F). This is consistent with results of the previous study that ARA55 was detected only in cell lines derived from prostate stroma.[31] In most of the 43 specimens studied, the expression level of ARA55 was lower in the stroma in regions of cancerous foci however quantification was not possible because of the scanty nature of stroma in cancerous foci.

Increased Expression of Ran/ARA24 and PIAS1 in Prostate Tumor Tissues

The 24-kDa protein Ran/ARA24 belongs to the superfamily of GTP binding proteins that use a structurally conserved G domain as a molecular switch for cycling between the GDP- and GTP-bound states.[32] Ran/ARA24 has been clearly implicated in the two-way traffic of macromolecules between the nucleus and the cytoplasm[33] and in microtubule assembly and spindle formation in cells in M phase. Recently, it has been shown that Ran/ARA24 physically interacts with the polyglutamine region of AR and enhances AR-dependent transcription.[34] Our in situ hybridization results for

normal tissue showed only a low level of Ran/ARA24 mRNA expression that was present mainly in epithelial cells (Figure 2A, and B). Comparison of Ran/ARA24 expression in normal and tumor tissues found overexpression (Figure 2, C and D vs. A and B, Table 3) in 81% of the tumor specimens, with an average increase of 4.6(+/-1.1)-fold and no change in 19% of the specimens. More dramatic changes (>5-fold) were observed in 35% of the tumor specimens (Table 3). However, these changes did not correlate with prostate tumor grade (Gleason score) by nonparametric Kruskal-Wallis ANOVA analysis and by the chi-square test when cases were further grouped according to changes of less than 2-fold and greater than 2-fold.

PIAS1 has been identified as a factor that binds to Stat1 (signal transducer and activator of transcription 1) and inhibits STAT-mediated signaling by interfering with the DNA binding of Stat1.[35] PIAS1 has also been identified as a coactivator for AR-, ER- and PR-dependent transcription.[36,37] In the current study, we observed higher PIAS1 expression levels in 33% of the tumor cases, with an average of 3.8-fold increase(Table 3). Although the percentage of cases showing an increase is significantly higher for Ran/ARA24 (81%) than for PIAS1 (33%), there is an 80% concordance between the increase for Ran/ARA24 and PIAS1. The PIAS1 expression patterns also did not correlate with prostate tumor grades by nonparametric Kruskal-Wallis ANOVA analysis and by the chi-square test.

Lower expression of ELE1/ARA70 in Prostate Cancer

ELE1/ARA70 was identified first as a factor involved in the activation of the RET proto-oncogene in thyroid neoplasia[38] and later as a ligand-dependent transcriptional cofactor for AR.[17] Our in situ RNA hybridization assays showed that ELE1/ARA70, like AR, is expressed at high levels (and predominantly in epithelial cells) in normal tissue (Figure 3, A and B). However, ELE1/ARA70 expression was dramatically lower in prostate tumor tissues (Figure 3, C and D versus A and B). Expression was decreased 2- to 5-fold in 42% of the cases and 5- to 30-fold in 38% of cases (Table 3), with an average decrease of 7.5(+/-1.4)-fold. We further observed that, in cases with increased expression of RAN/ARA24 and reduced expression of ELE1/ARA70, 70% of the cases showed reciprocal changes, indicating opposite effects of these coactivators in cancer. No obvious correlation between ELE1/ARA70 expression and prostate tumor grade was observed by nonparametric Kruskal-Wallis ANOVA analysis or by the chi-square test when cases were grouped according to changes less than two-fold and greater than two-fold.

AR Coactivator Expression in High Grade Prostate Intraepithelial Neoplasia (HGPIN)

High grade PIN (HGPIN) is thought to be a prostate cancer precursor lesion as a result of abundant evidence based on morphological, topographical, immunohistochemical and molecular

studies. HGPIN was identified in the majority of our cases (40 of 43 cases), either within or away from cancerous foci. A comparative analysis of AR cofactor expression patterns in HGPIN located adjacent to the prostate cancer region showed changes similar to those observed in prostate cancer. These changes included enhanced Ran/ARA24 expression (Figure 2, E and F versus A and B) and decreased ELE1/ARA70 expression (Figure 3, E and F versus A and B). We did not observe a significant difference in coactivator expression according to location of HGPIN. These results support the concept that HGPIN is a precursor of prostate cancer and further indicate that abnormal expression of Ran/ARA24 and ELE1/ARA70 may be involved in prostate tumor initiation.

Suppression of Human Prostate Cancer Cell Proliferation and Colony Formation by ELE1/ARA70

The decreased expression of ELE1/ARA70 in prostate cancer suggest that this cofactor might negatively regulate prostate cell growth and proliferation. We therefore tested the ability of ELE1/ARA70 gene to suppress the growth of prostate tumor cells, using the metastatic prostate cancer cell line LNCaP, which expresses reduced levels of ELE1/ARA70 compared with normal primary prostate epithelial cells.[40] Colony formation was suppressed by ELE1/ARA70 but not by the vector control (Figure 4). The colonies were small (containing a few cells), even after 1 month of G418 selection (data not shown). These results indicate that ELE1/ARA70 suppresses tumor cell proliferation and colony formation and

suggest that it may be a tissue differentiation factor or a potential tumor suppressor.

Discussion

Given the diverse functions of AR in different tissues, the large number of AR cofactors may provide means for cell- and promoter-specific regulation of AR activity.[1,2] Most cofactors are not receptor specific but also regulate the activity of many nuclear receptors as well as unrelated transcription factors.[3] Furthermore, many cofactors are components of multiprotein complexes that have overlapping functions and nuclear receptor-binding sites.[41] The challenge is to identify cofactors involved in AR function in the prostate, particularly in prostate growth and prostate cancer progression. Our results demonstrate the heterogeneous expression and functions of AR cofactors in the prostate. Significantly, we observed increased expression of PIAS1 and Ran/ARA24 and decreased expression of ELE1/RAR70 both in prostate cancer tissues and in high-grade PIN, relative to normal prostate tissue. Furthermore, our in vivo studies using malignant prostate cell line raise the possibility that ELE1/RAR70 might be a tumor suppressor.

AR and Some Cofactors Are Relatively Constant in Benign and Malignant Prostate Tissues

Enhanced AR activity has been correlated with prostate cancer formation and progression.[42] It also has been proposed that either AR gene mutation or AR gene amplification may enhance AR activity, thus promoting tumorigenesis or leading to androgen-independent prostate cancer.[1,43] However, the relatively low incidence of AR

mutation and amplification in primary prostate cancer suggests other causes. Consistent with this possibility, our in situ analyses have revealed that the levels and patterns of AR expression do not change significantly in primary prostate tumors of different grades. Downregulation of SRC1, one of general nuclear receptor cofactors, is associated with tamoxifen resistance in breast neoplasms.[44] However, we did not detect a significant change in expression of SRC1 mRNA in prostate tumor tissue relative to normal tissue. TRAP220 was expressed in both epithelial and stromal cells, and the levels were not different in prostate cancer and benign prostate tissues. This might reflect rather broad functions of TRAP220 for various nuclear receptors and other activators.[41]

Changed Expression of AR cofactors in Prostate Tumor

Recent studies have shown that various coactivators can bind to AR and augment the AR transcription activity in a ligand-dependent fashion.[1,2] Therefore, the activity of cofactors might contribute to enhanced AR activity in primary prostate cancer. Our study shows that expression levels of Ran/ARA24 and PIAS1 are significantly higher in prostate tumor tissue compared with nonneoplastic prostate tissue. The higher levels of Ran/ARA24 and PIAS1 may contribute to overproliferation of prostate tumor cells. PIAS1 belongs to a family of PIAS proteins that, consistent with present results, are able to coactivate steroid receptor-dependent transcription.[36,37] In contrast to increased expression of RAN/ARA24 in 81% of prostate tumor cases, enhanced expression of PIAS1 was

observed only in 33% of the cases. These differences may reflect the involvement of different pathways for Ran/ARA24 and PIAS1 as well as the relative efficiencies in contribution to cancer formation.

Interestingly, PIAS1 was first cloned as a protein that inhibited Stat1,[36] which has been suggested to have an antioncogenic effect.[45] This correlates with our findings that the level of PIAS1 is increased, possibly to revert the proapoptic activity of Stat1, in prostate tissue. Further investigations will be needed to determine whether high levels of PIAS1 promote prostate cell proliferation through the Stat1 pathway, the AR pathway, or both pathways. Similar to PIAS1, Ran/ARA24 is also involved in nuclear translocation and chromatin organization. It is however unclear which pathways are affected by the enhanced expression of RAN/ARA24.

Previous studies demonstrated that ELE1/ARA70 can serve as a coactivator of AR, ER, and PR.[16,17,46-49] Here we report a downregulation of ELE1/ARA70 expression in prostate cancer compared with levels expressed in non-neoplastic prostate tissues. Consistent with our observations, ELE1/ARA70 expression is reduced in prostate cancer cell lines relative to primary cells from benign prostate epithelium primary cells.[40] These observations suggest that ELE1/ARA70 may be involved in the development or progression of prostate cancer, particularly with respect to loss of androgen responsiveness. Overexpression of ELE1/ARA70 in a prostate cancer cell line suppresses cell proliferation and colony

formation, suggesting that it might be a tumor suppressor or involved in the expression of genes required for prostate cell differentiation. AR in LNCaP cell harbors a mutation (codon 877, Thr to Ala) in the hormone-binding domain. This mutation confers an altered ligand-binding specificity.

The study of cofactor expression in prostate cancer should be of great importance for understanding AR function in prostate tumorigenesis and progression. A shift in the levels of various AR cofactors may influence the state of differentiation or proliferation of the prostate, possibly through the regulation of different AR responsive genes.

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Figure legend

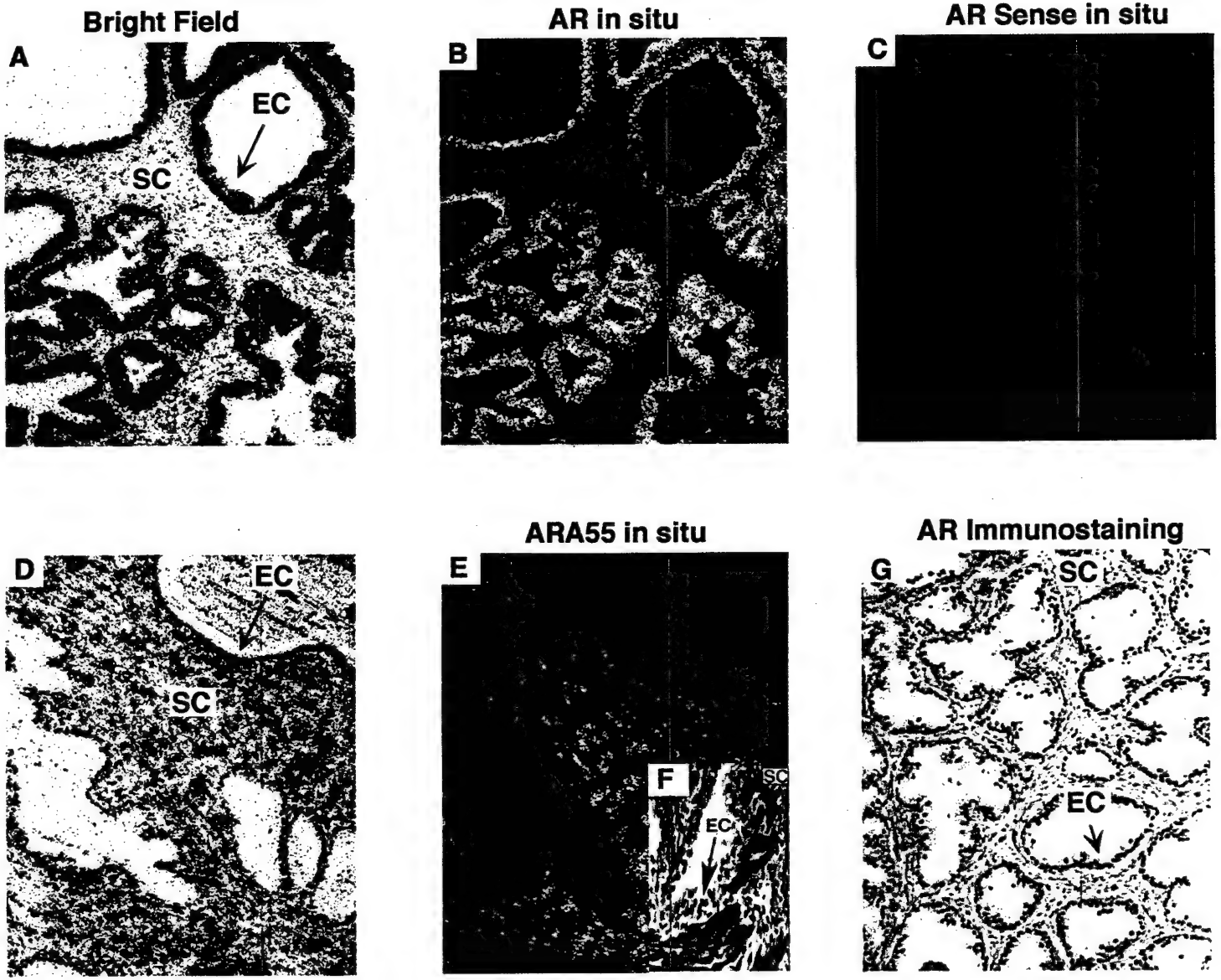
Figure 1. Expression of AR and ARA55 in prostate tissues. Panels **A** and **D** show bright field (emulsion-coated) of AR and ARA55, respectively. Panels **B** and **E** show dark field of the same slide areas. Panels **B** and **E** were hybridized with AR and ARA55 antisense probes, respectively, and panel **C** was hybridized with AR sense probes. Panel **F** and **G** shows an immonohistochemical analysis of ARA55 and AR in human prostate tissues. All slides were emulsion-coated, except panels **F** and **G**.

Figure 2. Increased expression of Ran/ARA24 in prostate cancer tissues. Left (**A, C, E**) and right panels (**B, D, F**) show bright field and dark fields of the same areas of slides, respectively. Panels **A-B**, **C-D**, and **E-F** show normal prostate, prostate tumor, and PIN tissues, respectively. All slides are emulsion-coated.

Figure 3. Decreased expression of ELE1/ARA70 in prostate cancer tissues. Left and right panels show bright and dark fields of the same slide areas, respectively. Panels **A-B**, **C-D** and **E-F** show normal prostate, prostate tumor and PIN tissues, respectively (**A**, **C**, and **E** were emulsion-coated slides).

Figure 4. Growth suppression of the prostate tumor cells by ELE1/ARA70. LNCaP prostate cancer cells were transfected with 4 μg of pcDNA3.1 (vector) or pcDNA-ELE1/ARA70 and selected for plasmid-containing cells with G418 for 4 weeks. Surviving cells

were then fixed and stained with crystal violet. Colonies were counted and the data are presented as histograms.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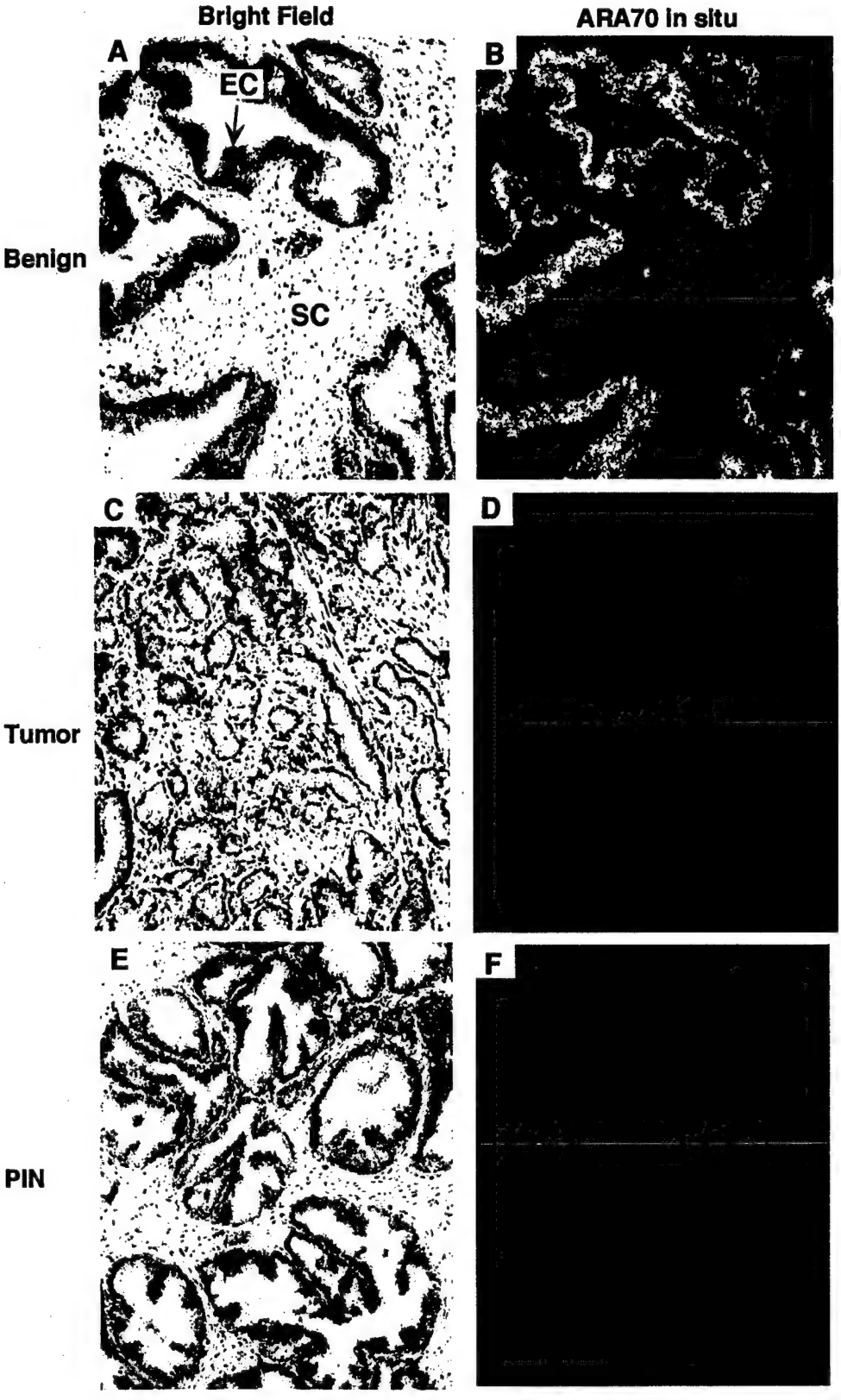
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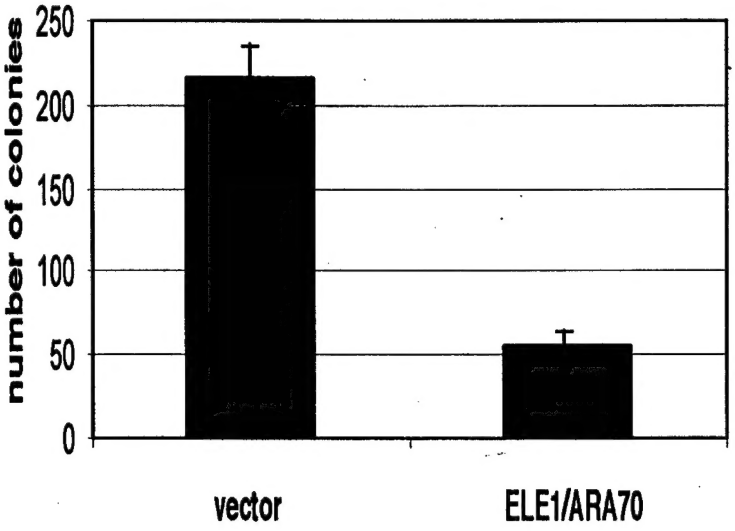
number of colonies
250
200
150
100
50
0
vector
ELE1/ARA70

Table 1: List of oligonucleotide primers used for PCR

Protein	Primer	Sequence (5'- 3')	Size (bp)
AR	T3/2263 T7/2762	accaatgtcaactccaggatgct cttcactgggtgtggaaatagatg	499
Ran/ARA24	T3/22 T7/627	caggtccagttcaaacttgtattggtt gagagcagttgtctgagcaacct	605
ARA54	T3/863 T7/1337	ttgcccgttatgaccgc aaatgtttgtaagggtttgctctagag	474
ARA55	T3/-20 T7/588	ctggagactaccacctcgacatg actgcagcagccccagc	608
ELE1/ARA70	T3/1344 T7/1835	tgagcctgagaagcataaagattc acatctgtagaggagttcgatataac	491
TMF1/ARA160	T3/477 T7/1058	ttcaggggaaactctggcag tatcccttgcctgacaattcatcat	581
PIAS1	T3/7 T7/645	gacagtgcggaactaaagcaaatg gaagtgatcttcttgtggacaactggt	638
SRC1	T3/3235 T73734	tatcagtcaccagacatgaagg ggttattcagtcagtagctgctg	499
TRAP220	T3/172 T7/800	ttggtcagctgtttggagacat ttgtacacagcagatgttccttca	628

T3 primers contain the T3 RNA polymerase promoter sequence gcaattaaccctcactaaaggg at and T7 primers contain the T7 RNA polymerase promoter sequence cgtaatacgactcactataggg at the 5' ends. Numbers indicate position of the primers' 5' ends on the cDNA sequences (the A of the ATG translation start codon was arbitrarily given the number 1).

Table 2: Summary of in situ hybridization data for AR and the eight examined cofactors

Factors	Stroma	Epithelium	
		Benign	Tumor
AR	++	+++	+++
SRC1	+	+	+
TMF1/ARA160	+/-	+	+
TRAP220	+/-	+	+
ARA55	++	-	-
ARA54	+/-	+	+
Ran/ARA24	+/-	+	+ + +
PIAS1	+/-	+	+ + +
ELE1/ARA70	-	+ + +	+

– indicates undetectable levels of expression. +/-, +, ++, and +++ indicate slightly above background, low, moderate, and high levels of expression, respectively.

Table 3: Quantification of PIAS1, Ran/ARA24, and ELE1/ARA70 expression

	PIAS1		Ran/ARA24			ELE1/ARA70		
fold	< 2	2 - 7.5	< 2	2 - 5	5 - 20	< 2	2 - 5	5 - 30
cases	24	12	8	20	15	9	18	16
%	67	33	19	46	35	20	42	38

Table 3: Quantitation of PIAS1, Ran/ARA24 and ELE1/ARA70 expression

	PIAS1		Ran/ARA24			ELE1/ARA70		
fold	< 2	2 - 7.5	< 2	2 - 5	5 - 20	< 2	2 - 5	5 - 30
cases	24	12	9	20	15	9	18	16
%	66	33	21	45	34	20	42	38